

**Biological Test Method:**  
**Test for Survival and Growth in Sediment**  
**Using the Larvae of Freshwater Midges**  
**(*Chironomus tentans* or *Chironomus riparius*)**



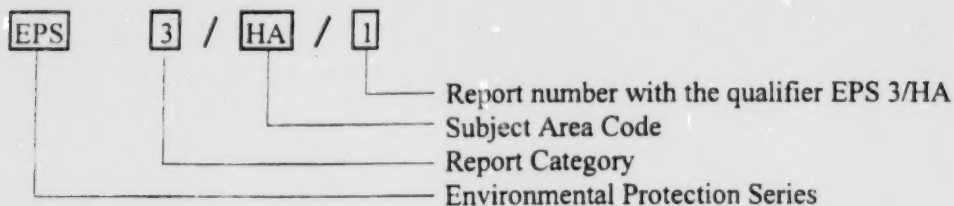
Environment  
Canada

Environnement  
Canada

Canada

# ENVIRONMENTAL PROTECTION SERIES

Sample Number:



## Categories

- 1 Regulations/Guidelines/Codes of Practice
- 2 Problem Assessments and Control Options
- 3 Research and Technology Development
- 4 Literature Reviews
- 5 Surveys
- 6 Social, Economic and Environmental Impact Assessments
- 7 Surveillance
- 8 Policy Proposals and Statements
- 9 Manuals

## Subject Areas

- |            |                                    |
|------------|------------------------------------|
| <b>AG</b>  | Agriculture                        |
| <b>AN</b>  | Anaerobic Technology               |
| <b>AP</b>  | Airborne Pollutants                |
| <b>AT</b>  | Aquatic Toxicity                   |
| <b>CC</b>  | Commercial Chemicals               |
| <b>CE</b>  | Consumers and the Environment      |
| <b>CI</b>  | Chemical Industries                |
| <b>FA</b>  | Federal Activities                 |
| <b>FP</b>  | Food Processing                    |
| <b>HA</b>  | Hazardous Wastes                   |
| <b>IC</b>  | Inorganic Chemicals                |
| <b>MA</b>  | Marine Pollutants                  |
| <b>MM</b>  | Mining and Ore Processing          |
| <b>NR</b>  | Northern and Rural Regions         |
| <b>PF</b>  | Paper and Fibres                   |
| <b>PG</b>  | Power Generation                   |
| <b>PN</b>  | Petroleum and Natural Gas          |
| <b>RA</b>  | Refrigeration and Air Conditioning |
| <b>RM</b>  | Reference Methods                  |
| <b>SF</b>  | Surface Finishing                  |
| <b>SP</b>  | Oil and Chemical Spills            |
| <b>SRM</b> | Standard Reference Methods         |
| <b>TS</b>  | Transportation Systems             |
| <b>TX</b>  | Textiles                           |
| <b>UP</b>  | Urban Pollution                    |
| <b>WP</b>  | Wood Protection/Preservation       |

New subject areas and codes are introduced as they become necessary. A list of EPS reports may be obtained from Environmental Protection Publications, Environmental Protection Service, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada.



**Biological Test Method:  
Test for Survival and Growth in Sediment  
Using Larvae of Freshwater Midges  
(*Chironomus tentans* or  
*Chironomus riparius*)**

Method Development and Application Section  
Environmental Technology Centre  
Environment Canada  
Ottawa, Ontario

Report EPS 1/RM/32  
December 1997

## Canadian Cataloguing in Publication Data

Main entry under title:

Biological test method. Test for survival and growth in sediment using larvae of freshwater midges (*Chironomus tentans* or *Chironomus riparius*)

(Report: EPS 1/RM/32)

Issued also in French under title: Méthode d'essai biologique. Essai de survie et de croissance des larves dulcicoles chironomes (*Chironomus tentans* ou *Chironomus riparius*) dans les sédiments.

Includes bibliographical references.

ISBN 0-660-16905-3

Cat. No. En49-24/1-32E

1. Diptera -- Toxicology -- Canada.
2. Chironomus -- Toxicology -- Canada.
3. Estuarine sediments -- Toxicology -- Canada.
4. Environmental monitoring -- Canada.
5. Toxicity testing -- Methodology -- Canada
- I. Environmental Technology Centre (Canada). Method Development and Application Section.
- II. Canada. Environment Canada.
- III. Series: Information report (Canada. Environment Canada); EPS 1/RM/32.

QL531.B54 1998

595.772

C98-980156-X



## **Readers' Comments**

---

Comments regarding the content of this report should be addressed to:

Richard Scroggins  
Method Development and Application Section  
Environmental Technology Centre  
Environment Canada  
3439 River Road South  
Gloucester, Ontario  
K1A 0H3

Cette publication est aussi disponible en français. Pour l'obtenir, s'adresser à:

Publications de la Protection de l'environnement  
Environnement Canada  
Ottawa (Ontario)  
K1A 0H3

## **Review Notice**

---

This report has been reviewed by the staff of the Environmental Technology Advancement Directorate, Environment Canada, and approved for publication. Mention of trade names or commercial products does not constitute recommendation or endorsement by Environment Canada for use.



## Abstract

---

*Methods recommended by Environment Canada for performing sediment toxicity tests using midge larvae (Chironomus tentans or C. riparius) are described in this report. The endpoints for the test are survival and weight of chironomid larvae at the end of a 10-day assay.*

*This 10-day sediment toxicity test is conducted at  $23 \pm 1$  °C in 300-mL glass beakers or jars containing a 100-mL layer of sediment and 175 mL of overlying water. A minimum of five test chambers, each containing 10 C. tentans or 10 C. riparius, are normally used to replicate each treatment. The test may be run as a single-concentration assay (e.g., using undiluted samples of field-collected sediment), or as a multi-concentration assay (e.g., a spiked sediment test with chemical/sediment mixtures) to determine the threshold of effect. Third instar C. tentans, or first instar C. riparius, are used to start the test.*

*The test may be performed either as an intermittent-renewal assay, or as a static (i.e., no renewal) toxicity test. If the renewal option is chosen, twice daily renewal of the overlying water in each test chamber is required, and the overlying water is normally not aerated. If the static option is chosen, the overlying water is aerated. Using either test option, the larvae are fed ground tropical fish food flakes, either as a daily ration of 6.0 mg dry solids per feed or four times only (nonconsecutive days) with 15.0 mg dry solids per feed. Selection of either option depends on the objectives of the study and perhaps also on regulatory guidelines or requirements.*

*General or universal conditions and procedures are outlined for test preparation and performance. Additional conditions and procedures are stipulated that are specific to the intended use of the test. This test is suitable for measuring and assessing the toxicity of samples of field-collected sediment, sludge, or similar particulate material, or of sediment spiked (mixed) in the laboratory with chemical(s) or chemical substance(s), contaminated sediment, or other particulate material. Instructions and requirements are included on test facilities, sample collection, handling and storing samples, culturing test organisms, preparing sediment or spiked-sediment mixtures and initiating tests, specific test conditions, appropriate observations and measurements, endpoints and methods of calculation, and the use of reference toxicants.*

## Résumé

---

*Le présent rapport décrit les méthodes que recommande Environnement Canada pour les essais de mesure de la toxicité des sédiments à l'aide de larves de chironomes (Chironomus tentans ou C. riparius). Les paramètres de mesure sont la survie et le poids des larves au bout des 10 journées de l'essai.*

*L'essai se déroule à  $23 \pm 1$  °C, dans des bechers ou des jarres de verre de 300 mL renfermant 100 mL de sédiment recouvert de 175 mL d'eau. Il faut normalement cinq de ces récipients au moins, renfermant chacun 10 C. tentans ou C. riparius, par variante de l'expérience. L'essai peut porter sur une seule concentration (p. ex. des échantillons non dilués de sédiment prélevés sur le terrain) ou sur une gamme de concentrations (p. ex. sédiment enrichi de différentes concentrations de mélanges de produits chimiques et de sédiment) afin de déterminer le seuil à partir duquel se manifeste l'effet. Au début de l'essai, les larves sont du troisième stade de C. tentans ou du premier stade de C. riparius.*

*Pendant l'essai, on peut renouveler ou non l'eau surnageante. Dans le premier cas, on doit la renouveler deux fois par jour dans chaque récipient, sans normalement l'aérer. En conditions statiques (sans renouvellement) cependant, on aère l'eau. Quelle que soit l'option retenue, on nourrit les larves de flocons moulus de nourriture pour poissons tropicaux, soit journellement, à raison de 6,0 mg de matières solides par repas, soit quatre fois seulement (au cours de journées non consécutives) au moyen de 15,0 mg de matières solides par repas. Le choix du régime dépend de l'objectif de l'étude et peut-être aussi des lignes directrices ou des exigences réglementaires.*

*Le rapport expose les conditions et le mode opératoire généraux ou universels de la préparation de l'essai et de son exécution. S'y ajoutent des conditions et des modes opératoires propres à la finalité prévue de l'essai. Ce dernier permet de mesurer et d'évaluer la toxicité d'échantillons de sédiment, de boue ou de matières particulières semblables, prélevés sur le terrain, ou de sédiment enrichi (additionné) au laboratoire de substances ou de produits chimiques, de sédiment contaminé ou d'autres matières particulières. Le rapport renferme aussi des instructions et des exigences sur les installations servant à la réalisation des essais, sur le prélèvement des échantillons, leur manutention et leur entreposage, sur l'élevage des organismes d'essai, sur la préparation du sédiment ou des mélanges de sédiment et d'autres matières ainsi que sur la mise en branle des essais, les conditions expérimentales précises, les observations et les mesures convenables à faire, les paramètres de mesure et les méthodes de calcul de même que l'emploi de toxiques de référence.*

## Foreword

---

*This is one of a series of recommended methods for measuring and assessing the aquatic biological effects of toxic substances or materials. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

- *for use in EC aquatic toxicity laboratories;*
- *for testing that is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as might be required in a regulatory protocol or standard reference method.*

*The different types of tests included in this series were selected because of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on the toxicity to aquatic life of specific test substances or materials destined for or within the aquatic environment. Depending on the biological test method chosen, substances or materials to be tested for toxicity could include samples of chemical or chemical substance, effluent, elutriate, leachate, receiving water or, where appropriate, sediment or similar particulate material.*



## Table of Contents

---

|                                                          |       |
|----------------------------------------------------------|-------|
| <b>Abstract</b> .....                                    | v     |
| <b>Résumé</b> .....                                      | vi    |
| <b>Foreword</b> .....                                    | vii   |
| <b>List of Tables</b> .....                              | xiii  |
| <b>List of Figures</b> .....                             | xiii  |
| <b>List of Abbreviations and Chemical Formulae</b> ..... | xiv   |
| <b>Terminology</b> .....                                 | xvi   |
| <b>Acknowledgements</b> .....                            | xxiii |

### *Section 1*

|                                                          |   |
|----------------------------------------------------------|---|
| <b>Introduction</b> .....                                | 1 |
| 1.1 Background .....                                     | 1 |
| 1.2 Identification, Distribution, and Life History ..... | 5 |
| 1.3 Historical Use in Toxicity Tests .....               | 6 |
| 1.4 Laboratory Tolerance and Relative Sensitivity .....  | 8 |

### *Section 2*

|                                                    |    |
|----------------------------------------------------|----|
| <b>Test Organisms</b> .....                        | 12 |
| 2.1 Species and Life Stage .....                   | 12 |
| 2.2 Source and Acclimation .....                   | 12 |
| 2.3 Culturing .....                                | 13 |
| 2.3.1 General .....                                | 13 |
| 2.3.2 Facilities and Apparatus .....               | 14 |
| 2.3.3 Lighting .....                               | 16 |
| 2.3.4 Culture Water .....                          | 16 |
| 2.3.5 Temperature .....                            | 18 |
| 2.3.6 Dissolved Oxygen .....                       | 18 |
| 2.3.7 Culturing Substrate .....                    | 18 |
| 2.3.8 Food and Feeding .....                       | 19 |
| 2.3.9 Handling Organisms .....                     | 19 |
| 2.3.10 Establishing and Maintaining Cultures ..... | 19 |
| 2.3.11 Health Criteria .....                       | 20 |

### *Section 3*

|                                           |    |
|-------------------------------------------|----|
| <b>Test System</b> .....                  | 22 |
| 3.1 Facilities and Apparatus .....        | 22 |
| 3.2 Lighting .....                        | 24 |
| 3.3 Test Chambers .....                   | 24 |
| 3.4 Test and Control/Dilution Water ..... | 24 |
| 3.5 Control Sediment .....                | 25 |



*Section 4*

|                                                   |    |
|---------------------------------------------------|----|
| <b>Universal Test Procedures</b>                  | 26 |
| 4.1 Beginning the Test                            | 26 |
| 4.2 Test Conditions                               | 34 |
| 4.3 Dissolved Oxygen and Aeration                 | 35 |
| 4.4 Food and Feeding                              | 35 |
| 4.5 Observations and Measurements During the Test | 37 |
| 4.6 Ending a Test                                 | 38 |
| 4.7 Test Endpoints and Calculations               | 40 |
| 4.8 Tests with Reference Toxicant(s)              | 41 |

*Section 5*

|                                                                                                     |    |
|-----------------------------------------------------------------------------------------------------|----|
| <b>Specific Procedures for Testing Field-collected Sediment<br/>or Similar Particulate Material</b> | 46 |
| 5.1 Sample Collection                                                                               | 46 |
| 5.2 Sample Labelling, Transport, Storage,<br>and Analyses                                           | 48 |
| 5.3 Preparing Sample for Testing                                                                    | 50 |
| 5.4 Test Water                                                                                      | 51 |
| 5.5 Test Observations and Measurements                                                              | 51 |
| 5.6 Test Endpoints and Calculations                                                                 | 52 |
| 5.6.1 Variations in Design and Analysis                                                             | 54 |
| 5.6.2 Power Analysis                                                                                | 55 |

*Section 6*

|                                                                     |    |
|---------------------------------------------------------------------|----|
| <b>Specific Procedures for Testing Spiked Sediment</b>              | 57 |
| 6.1 Sample Properties, Labelling, and Storage                       | 57 |
| 6.2 Preparing Test Mixtures                                         | 58 |
| 6.3 Test and Control/Dilution Water                                 | 61 |
| 6.4 Test Observations and Measurements                              | 62 |
| 6.5 Test Endpoints and Calculations                                 | 62 |
| 6.5.1 Median Lethal Concentration (LC50)                            | 62 |
| 6.5.2 Inhibiting Concentration for a Specified Percent Effect (ICp) | 64 |
| 6.5.3 Hypothesis Testing (NOEC and LOEC)                            | 67 |

*Section 7*

|                                                     |    |
|-----------------------------------------------------|----|
| <b>Reporting Requirements</b>                       | 69 |
| 7.1 Minimum Requirements for a Test-specific Report | 70 |
| 7.1.1 Test Substance or Material                    | 70 |
| 7.1.2 Test Organisms                                | 70 |
| 7.1.3 Test Facilities                               | 70 |
| 7.1.4 Test Water                                    | 70 |



|                                                                                                                                                                         |                                         |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|-----|
| 7.1.5                                                                                                                                                                   | Test Method .....                       | 70  |
| 7.1.6                                                                                                                                                                   | Test Conditions and Procedures .....    | 70  |
| 7.1.7                                                                                                                                                                   | Test Results .....                      | 71  |
| 7.2                                                                                                                                                                     | Additional Reporting Requirements ..... | 72  |
| 7.2.1                                                                                                                                                                   | Test Substance or Material .....        | 72  |
| 7.2.2                                                                                                                                                                   | Test Organisms .....                    | 72  |
| 7.2.3                                                                                                                                                                   | Test Facilities and Apparatus .....     | 72  |
| 7.2.4                                                                                                                                                                   | Control Sediment and Test Water .....   | 72  |
| 7.2.5                                                                                                                                                                   | Test Method .....                       | 73  |
| 7.2.6                                                                                                                                                                   | Test Conditions and Procedures .....    | 73  |
| 7.2.7                                                                                                                                                                   | Test Results .....                      | 73  |
| References .....                                                                                                                                                        |                                         | 75  |
| <i>Appendix A</i>                                                                                                                                                       |                                         |     |
| Members of the Inter-Governmental Aquatic<br>Toxicity Group (as of July 1997) .....                                                                                     |                                         | 91  |
| <i>Appendix B</i>                                                                                                                                                       |                                         |     |
| Environment Canada, Environmental Protection Service,<br>Regional and Headquarters Offices .....                                                                        |                                         | 93  |
| <i>Appendix C</i>                                                                                                                                                       |                                         |     |
| Procedural Variations for Culturing<br><i>Chironomus</i> sp., as Described in Canadian,<br>United States, and European Methodology Documents .....                      |                                         | 94  |
| <i>Appendix D</i>                                                                                                                                                       |                                         |     |
| Procedural Variations for Sediment Toxicity<br>Tests Using <i>Chironomus</i> sp., as Described in<br>Canadian, United States, and European Methodology Documents .....  |                                         | 102 |
| <i>Appendix E</i>                                                                                                                                                       |                                         |     |
| Procedural Variations for Reference Toxicity<br>Tests Using <i>Chironomus</i> sp., as Described in<br>Canadian, United States, and European Methodology Documents ..... |                                         | 115 |
| <i>Appendix F</i>                                                                                                                                                       |                                         |     |
| General Systematics of <i>Chironomus tentans</i><br>(Fabricius, 1805) .....                                                                                             |                                         | 121 |

*Appendix G*

**General Systematics of *Chironomus riparius*  
(Meigen, 1804)**

127

*Appendix H*

**Logarithmic Series of Concentrations Suitable  
for Toxicity Tests**

131

## List of Tables

---

- 1 Checklist of Recommended Conditions and Procedures for Culturing Larval *Chironomus* sp. for Use in Sediment Toxicity Tests ..... 15
- 2 Checklist of Recommended Conditions and Procedures for Conducting Sediment Toxicity Tests using *Chironomus* sp. .... 27
- 3 Age and Head Capsule Width of *Chironomus tentans* and *Chironomus riparius* Instars Reared at 23°C ..... 33
- 4 Checklist of Recommended Conditions and Procedures for Conducting *Water Only* Reference Toxicity Tests using *Chironomus* sp. .... 42

## List of Figures

---

- 1 Considerations for Preparing and Performing Toxicity Tests Using *Chironomus* sp. and Various Types of Test Materials or Substances ..... 4
- 2 Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper ..... 65

## List of Abbreviations and Chemical Formulae

---

|                  |                                                               |
|------------------|---------------------------------------------------------------|
| ANOVA            | ... analysis of variance                                      |
| $\text{CaCl}_2$  | ... calcium chloride                                          |
| $\text{CaCO}_3$  | ... calcium carbonate                                         |
| $\text{CaSO}_4$  | ... calcium sulphate                                          |
| $\text{CdCl}_2$  | ... cadmium chloride                                          |
| $\text{CuSO}_4$  | ... copper sulphate                                           |
| cm               | ... centimetre(s)                                             |
| CV               | ... coefficient of variation                                  |
| d                | ... day(s)                                                    |
| DO               | ... dissolved oxygen (concentration)                          |
| EC50             | ... median effective concentration                            |
| g                | ... gram(s)                                                   |
| h                | ... hour(s)                                                   |
| ICp              | ... inhibiting concentration for a (specified) percent effect |
| KCl              | ... potassium chloride                                        |
| kg               | ... kilogram(s)                                               |
| L                | ... litre(s)                                                  |
| LC50             | ... median lethal concentration                               |
| LOEC             | ... lowest-observed-effect concentration                      |
| mg               | ... milligram(s)                                              |
| $\text{MgSO}_4$  | ... magnesium sulphate                                        |
| min              | ... minute(s)                                                 |
| mL               | ... millilitre(s)                                             |
| mS               | ... millisiemens                                              |
| MSD              | ... minimum significant difference                            |
| mm               | ... millimetre(s)                                             |
| NaCl             | ... sodium chloride                                           |
| $\text{NaHCO}_3$ | ... sodium bicarbonate                                        |
| NOEC             | ... no-observed-effect concentration                          |
| $\text{O}_2$     | ... oxygen                                                    |
| SD               | ... standard deviation                                        |
| s                | ... second                                                    |
| TOEC             | ... threshold-observed-effect concentration                   |
| TM (™)           | ... Trade Mark                                                |
| v:v              | ... volume-to-volume                                          |
| YCT              | ... yeast, Cerophyll™, and trout chow                         |
| °C               | ... degree(s) Celsius                                         |
| µg               | ... microgram(s)                                              |
| µm               | ... micrometre(s)                                             |
| µmhos/cm         | ... micromhos per centimetre                                  |
| µS               | ... microsiemen(s)                                            |

|        |                          |
|--------|--------------------------|
| $>$    | greater than             |
| $<$    | less than                |
| $\geq$ | greater than or equal to |
| $\leq$ | less than or equal to    |
| $\pm$  | plus or minus            |
| $\%$   | percentage or percent    |

## Terminology

---

Note: all definitions are given in the context of the procedures in this report, and might not be appropriate in another context.

### Grammatical Terms

*Must* is used to express an absolute requirement.

*Should* is used to state that the specified condition or procedure is recommended and ought to be met if possible.

*May* is used to mean "is (are) allowed to".

*Can* is used to mean "is (are) able to".

*Might* is used to express the possibility that something could exist or happen.

### General Technical Terms

*Acclimation* is physiological adjustment to a particular level of one or more environmental factors such as temperature. The term usually refers to controlled laboratory conditions.

*Antennal ratio* is the length of the long distal flagellar segment plus the apical segment (if present), divided by the combined length of the short basal flagellar segments. This ratio is measured on adult males only.

*Compliance* means in accordance with governmental permitting or regulatory requirements.

*Conductivity* is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valence and mobility, and on the solution's temperature. Conductivity is reported as micromhos per centimetre ( $\mu\text{mhos/cm}$ ) or as millisiemens per metre ( $\text{mS/m}$ );  $1 \text{ mS/m} = 10 \text{ } \mu\text{mhos/cm}$ .

*Flowthrough* describes apparatus or tests in which solutions or overlying water in culture or test chambers are/is renewed continuously by the constant inflow of a fresh solution.

*Intermittent renewal* describes apparatus or tests in which solutions or overlying water in culture or test chambers are/is renewed periodically, usually at the beginning of each 12-h or 24-h

period. Synonymous terms are *batch replacement*, *renewed static*, *renewal*, *static renewal*, *static replacement*, and *semistatic*.

*Leg ratio* is the length of the first tarsal segment (i.e., basitarsus) divided by the length of the tibia. This ratio is measured on the foreleg of adult males or females.

*Lux* is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux.

*Monitoring* is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality, or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water quality variables, or the collection and testing of samples of sediment for toxicity.

*Percentage (%)* is a concentration expressed in parts per hundred parts. With respect to test substances, ten percent (10%) represents ten units or parts of substance diluted with sediment or water to a total of 100 parts. Depending on the test substance, concentrations can be prepared on a weight-to-weight, weight-to-volume, or volume-to-volume basis, and are expressed as the percentage of test substance in the final sediment mixture or solution.

*pH* is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 indicating increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

*Photoperiod* is the duration of illumination and darkness within a 24-h day.

*Pretreatment* means treatment of a sediment sample, or portion thereof, before exposure of test organisms.

*Static* describes toxicity tests in which test solutions or overlying water are not renewed during the test.

## Terms for Test Materials or Substances

*Chemical* is, in this report, any element, compound, formulation, or mixture of a substance that might be mixed with, deposited in, or found in association with sediment or water.

*Clean sediment* is sediment that does not contain concentrations of any substance(s) causing discernible distress to the test organisms or reduces their survival or growth during the test.

*Contaminated sediment* is sediment containing chemical substances at concentrations that pose a known or potential threat to environmental or human health.

*Control* is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no added test material or substance. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., temperature, health of test organisms, or effects due to their handling).

*Control/dilution water* is the water used for preparing a series of concentrations of a test chemical, or that used as overlying water in a sediment toxicity test or as control water in a *water only* test with a reference toxicant. Control/dilution water is frequently identical to the culture and test (overlying) water.

*Control sediment* is *clean* sediment not containing concentrations of one or more contaminants that could affect the survival, growth, or behaviour of the test organisms. Control sediment might be natural sediment from an uncontaminated site, or formulated (reconstituted) sediment. This sediment must contain no added test material or substance, and must enable acceptable (i.e.,  $\geq 70\%$ ) survival of the test organisms during the test. The use of control sediment provides a basis for interpreting data derived from toxicity tests using test sediment(s), and also provides a base sediment for spiking procedures.

*Dechlorinated water* is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from solution.

*Deionized water* is water that has been purified by passing it through resin columns or a reverse osmosis system, for the purpose of removing ions such as  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ .

*Distilled water* is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

*Overlying water* is water placed over sediment in a test chamber.

*Pore water* (also called *interstitial water*) is the water occupying space between sediment particles.



*Reconstituted water* is high purity deionized or glass distilled water to which reagent grade chemicals have been added. The resultant synthetic fresh water should be free from contaminants and have the desired pH, alkalinity, and hardness characteristics.

*Reference sediment* is a field-collected sample of presumably *clean* sediment, selected for properties (e.g., particle size, compactness, total organic content) representing sediment conditions that closely match those of the sample(s) of test sediment except for the degree of chemical contaminants. It is often selected from a site uninfluenced by the source(s) of contamination but within the general vicinity of the sites where samples of test sediment are collected.

*Reference toxicant* is a standard chemical used to measure the sensitivity of the test organisms in order to establish confidence in the toxicity data obtained for a test material or substance. In most instances, a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical.

*Reference toxicity test* is a test conducted using a reference toxicant in conjunction with a sediment toxicity test, to appraise the sensitivity of the organisms at the time the test material or substance is evaluated, and the precision and reliability of results obtained by the laboratory for that chemical. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect. A reference toxicity test is most often performed in the absence of sediment (i.e., as a *water only* test), although it can also be conducted as a *spiked sediment* test.

*Sediment* is natural particulate material which has been transported and deposited in water and usually lies below water. The term can also describe a substrate that has been experimentally prepared (formulated) using selected particulate material (e.g., sand of particular grain size, bentonite clay, etc.) and within which the test organisms can burrow.

*Solid-phase sediment* (also called *whole* sediment) is the intact sediment used to expose the test organisms, not a form or derivative of the sediment such as pore water or a resuspended sediment.

*Spiked control sediment* is clean control sediment to which a test substance or material such as a chemical, a mixture of chemicals, drilling mud, contaminated dredge spoil, sludge, or contaminated sediment has been added experimentally, and mixed thoroughly to evenly distribute the substance throughout the control sediment.

*Spiked sediment* is any sediment (clean or contaminated) to which a test substance or material such as a chemical, a mixture of chemicals, drilling mud, contaminated dredge spoil, sludge, or contaminated sediment has been added experimentally, and mixed thoroughly to evenly distribute the substance or material throughout the sediment.

*Stock solution* is a concentrated solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water to prepare the required strengths of test solutions.

*Substance* is a particular kind of material having more or less uniform properties.

*Test sediment* is a field-collected sample of solid-phase sediment, taken from a site thought to be contaminated with one or more chemicals, and intended for use in the toxicity test. In some instances, the term also applies to any sediment sample or mixture of spiked sediment (including control and reference sediment) used in the test.

*Test water* is the water placed over the layer of sediment in the test chambers, i.e., *overlying* water. It also denotes the water used to manipulate the sediment, if necessary (e.g., for preparing formulated sediment or mixtures of spiked sediment, or for wet sieving), and that used as control/dilution water for *water only* tests with reference toxicants.

## Statistical and Toxicological Terms

*Acute* means within a short period (seconds, minutes, hours, or a few days) in relation to the life span of the test organism.

*Acute toxicity* means a discernible adverse effect (lethal or sublethal) induced in the test organisms within a short period of exposure (in this instance, <10 days) to a test substance.

*Chronic* means occurring during a relatively long period of exposure, usually a significant portion of the life span of the organism such as 10% or more.

*Chronic toxicity* implies long-term effects that are related to changes in metabolism, growth, reproduction, or ability to survive.

*Chronic value* is the geometric mean of the NOEC and LOEC in tests which have a chronic exposure. See also *TOEC* as a recommended term.

*Coefficient of Variation (CV)* is the standard deviation (SD) of a set of data divided by the mean, expressed as a percentage. It is calculated as:

$$CV (\%) = 100 \text{ SD} \div \text{mean.}$$

*Endpoint* means the variable(s) (i.e., time, reaction of the organisms, etc.) that indicate(s) the termination of a test, and also means the measurement(s) or derived value(s) that characterize the results of the test (e.g., LC50, ICp, NOEC, LOEC).

*ICp* is the inhibiting concentration for a (specified) percentage effect. It represents a point estimate of the concentration of test material or substance that causes a designated percent impairment in a quantitative biological function such as growth. For example, an IC25 could be the concentration estimated to cause a 25% reduction in dry weight attained at the end of the test by the test organisms relative to that in the control. This term should be used for any toxicological test that measures a continuously variable effect, such as dry weight at test end, reproduction, or respiration.

*LC50* is the median lethal concentration, i.e., the concentration of substance or material in sediment (e.g., mg/kg) or water (e.g., mg/L) estimated to be lethal to 50% of the test organisms. The LC50 and its 95% confidence limits are usually derived by statistical analysis of mortalities in five or more test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 96-h LC50 for a *water only* reference toxicity test, or 10-d LC50 for a *survival-and-growth* sediment toxicity test).

*Lethal* means causing death by direct action. Death of *Chironomus* sp. is defined as the cessation of all visible signs of activity indicating life.

*LOEC* is the lowest-observed-effect concentration. This is the lowest concentration of a test substance or material to which organisms are exposed, that causes observed and statistically significant sublethal effects on the organism. For example, the LOEC might be the lowest concentration at which the dry weight of exposed organisms at test end was significantly less than that in the control groups.

*Minimum Significant Difference (MSD)* means the difference between values for individual treatments (in this test with midge larvae, the difference in average weights or average mortality) that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by certain statistical tests including *Dunnett's multiple-range test*, a standard statistical procedure.

*NOEC* is the no-observed-effect concentration. This is the highest concentration of a test substance or material to which organisms are exposed, that does not cause any observed and statistically significant sublethal effects on the organism. For example, the NOEC might be the highest test concentration at which an observed variable such as dry weight at test end is not decreased significantly from weight in the control groups.

*Precision* refers to the closeness of repeated measurements of the same quantity to each other, i.e., the degree to which data generated from replicate measurements differ. It describes the degree of certainty around a result, or the tightness of a statistically derived endpoint such as an ICp.

*Replicate* refers to a single test chamber containing a prescribed number of organisms in either one concentration of the test material or substance, or in the control or reference treatment(s). In a toxicity test comprising five replicate samples of undiluted field-

collected sediment taken from each of four sites (including a reference site) plus replicate samples of control sediment, 25 test chambers would be used. For each treatment (i.e., for a particular sediment-collection site or, in the case of a multi-concentration test with contaminant-spiked sediment), there would normally be a minimum of five test chambers or replicates. A replicate is an independent test unit; therefore, any transfer of organisms or test material from one replicate to another would invalidate the test.

*Sublethal* means detrimental to the organism, but below the level that directly causes death within the test period.

*Sublethal effect* is an adverse effect on an organism, below the level which directly causes death within the test period.

*TOEC* is the threshold-observed-effect concentration. It is calculated as the geometric mean of NOEC and LOEC. In some other countries, the concentration calculated in this way might be called the MATC (maximum acceptable toxicant concentration). *Chronic value* and *subchronic value* are alternative terms that have been used elsewhere and might be appropriate depending on the duration of the test.

*Toxicity* is the inherent potential or capacity of a substance or material to cause adverse effect(s) on living organisms. The effect(s) could be lethal or sublethal.

*Toxicity test* is a procedure for determining the effect of a substance or material on a group of selected organisms (e.g., *Chironomus tentans* or *C. riparius*), under defined conditions. An aquatic toxicity test usually measures: (a) the proportions of organisms affected (*quantal*); and/or (b) the degree of effect shown (*quantitative* or *graded*), after exposure to a specific test substance or material (e.g., a sample of sediment) or mixture thereof (e.g., a chemical/sediment mixture).

*Water renewal* describes the renewal of the overlying water in test chambers, on a regular and timed basis (e.g., one or two times daily) throughout the test. This may be done manually or using an automated system which enables intermittent renewal of overlying water at a fixed rate.

## Acknowledgements

---

*This biological test method was co-authored by D.J. McLeay (McLeay Environmental Ltd., Victoria, BC) and K.E. Day (Rivers Research Branch, National Water Research Institute, Environment Canada, Burlington, ON).*

*Initial methodology development work and testing support within Canada were provided by K. Day, P. Gillis, P. Hamr, S. Kirby, and D. Milani of Environment Canada's National Water Research Institute, Canada Centre for Inland Waters (Burlington, ON). P. Hamr is thanked for his illustrations and descriptions of the general systematics of *C. tentans* and *C. riparius*. Statistical guidance herein was written or reviewed and improved by J.B. Sprague (Sprague Associates Ltd., Salt Spring Island, BC).*

*The report is based on existing reports (published or otherwise) that describe procedures and conditions used in the United States, Canada, and Europe for culturing *C. tentans* or *C. riparius*, and for conducting sediment toxicity tests using midge larvae representing either species. In particular, the specific procedures and conditions for culturing and testing *C. tentans* recommended by the United States Environmental Protection Agency (USEPA, 1994a), together with subsequent intra- and interlaboratory studies with *C. riparius* undertaken by Canadian and United States researchers (Milani et al., 1996), guided the development of the present EC biological test method. With regard to these method development and validation studies (Milani et al., 1996), we gratefully acknowledge the participation and contribution of the following individuals and organizations: D. Milani and S. Kirby (National Water Research Institute, Environment Canada, Burlington, ON); K. Doe (Environment Canada, Moncton, NB); K. Holtze (B.A.R. Environmental Inc., Guelph, ON); A. Putt (Springborn Laboratories Inc., Wareham, MA); P. Riebel (Beak Consultants Ltd., Dorval, PQ); and G. van Aggelen (Environment Canada, North Vancouver, BC).*

*R.P. Scroggins (Method Development and Application Section, Environmental Technology Centre, Environment Canada, Ottawa, ON) acted as Scientific Authority and provided technical input and direction throughout the work. Members of the Inter-Governmental Aquatic Toxicity Group (IGATG, Appendix A) participated in the development and review of this document and are thanked accordingly. Special acknowledgement is made of the many useful comments provided by each member of the Environment Canada (EC) committee of scientific experts responsible for the document's initial and final reviews: G.T. Ankley (USEPA, Duluth, MN); D. Bedard (Ontario Ministry of Environment and Energy, Etobicoke, ON); G.A. Burton, Jr. (Wright State University, Dayton, OH); M. Hamer (Zeneca Agrochemicals, Berkshire, U.K.); C.G. Ingersoll (U.S. Geological Survey, Columbia, MO); K. Liber (University of Wisconsin, Superior,*



WI); C. Naylor (University of Sheffield, Sheffield, U.K.); and D. Pascoe (University of Wales College of Cardiff, Cardiff, U.K.). Three of the principal authors of USEPA (1994a), namely, C.G. Ingersoll, G.T. Ankley, and G.A. Burton, served on Environment Canada's committee of scientific experts.

*In addition to the members of the Environment Canada scientific advisory committee who contributed to the development of this biological test method, the following people reviewed the final draft and provided many useful comments: D. Bedard and G. Westlake (Ontario Ministry of Environment and Energy, Etobicoke, ON); J. Black (EA Eng. Sci. Tech. Inc., Sparks, MD); R.L. Breton (Environment Canada, Ottawa, ON); R.J. Casey (Alberta Environmental Centre, Vegreville, AB); P. Dohmen (BASF, Limburgerhof, Germany); P. Downey (TRAC Laboratories Inc., Denton, TX); C.W. Hickey (NIWA Ecosystems, Hamilton, New Zealand); R.A. Hoke (SAIC, Hackensack, N.J.); D.R. Oliver (Agriculture Canada, Ottawa, ON); F. Quiniou (IFREMER, Plouzane, France); C.J. Roghair (RIVM, Netherlands); and P.V. Winger (Univ. of Georgia, Athens, GA).*

## Section 1

---

# Introduction

## 1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to determine and monitor the toxic effects of discrete substances or complex mixtures that might be harmful to indigenous aquatic life in the environment (water and sediment). The results of toxicity tests can be used to determine the need for control of discharges, to set effluent standards, and for research and other purposes. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, Environment Canada and the Inter-Governmental Aquatic Toxicity Group (IGATG) (Appendix A) proposed in 1987 that a set of standardized aquatic toxicity tests be developed, that would be broadly acceptable for use in Canada. At that time, it was decided that a battery of federally approved biological test methods was required that would measure different toxic effects using different test substances or materials (e.g., samples of chemical or chemical substance, effluent, receiving water, or sediment) and organisms representing different trophic levels and taxonomic groups (Sergy, 1987). As part of this ongoing undertaking, Environment Canada and the IGATG recommended that a number of standardized sediment toxicity tests be developed for routine use in preventing, appraising, remediating, and managing contaminated sediment.

In the aquatic environment, sediment provides habitat for many organisms, while

being a major repository for many of the more persistent chemicals that are introduced to surface waters. There is growing evidence of environmental deterioration, including adverse effects on aquatic organisms in or near sediment, even when federal or provincial water quality criteria are not exceeded. Other evidence shows that sediment can be contaminated with high concentrations of chemicals of toxic concern, yet a diverse community of benthic or epibenthic organisms is present and no harm to exposed aquatic life can be demonstrated. Sediment toxicity tests are cost-effective tools for determining whether contaminants in sediment are harmful to benthic or epibenthic organisms as well as those frequenting the overlying water column. These tests can be used for various purposes, including:

- determining the spatial and temporal distribution of contaminants in sediment that are toxic to benthic or epibenthic organisms;
- measuring the toxicity of dredged or other material being considered for disposal to surface waters;
- ranking aquatic environments adjacent to industrial sites for quality and/or cleanup requirements;
- appraising the environmental integrity of approved disposal sites;
- estimating the effectiveness of management or remediation practices;

- investigating interactions of environmental relevance between contaminants in sediment and overlying water;
- comparing the relative sensitivities of various benthic and epibenthic organisms; and
- determining the relationship between toxic effects and bioavailability for contaminants in sediment.

In the laboratory, results of toxicity tests using samples of clean sediment spiked with different concentrations of contaminants can be used to assess cause-and-effect relationships between chemicals and biological responses. Environmental appraisal of spatial (horizontal and/or vertical) and temporal sediment quality are most effective if they integrate a battery of appropriate sediment toxicity tests with sediment chemistry (including bulk sediment and porewater analyses) and biological surveys.

Environment Canada's regional laboratories (Appendix B) are actively involved in studies to develop and standardize biological test methods for measuring the toxicity of samples of contaminated sediment. A sediment toxicity test using one or more of six species of marine or estuarine amphipods common to Canadian Pacific or Atlantic coastal waters has been developed (EC, 1992a), and a rapid procedure is available for measuring the toxicity of samples of sediment, pore water, or elutriate using luminescent bacteria (EC, 1992b). A test for survival and growth in sediment, using the freshwater amphipod *Hyaletta azteca*, has been published by Environment Canada (EC,

1997a). Environment Canada is also developing a test for survival and growth in sediment that uses cultured marine or estuarine polychaete worms common to Canadian coastal waters (EC, 1997b). Other Environment Canada test methods can be used for measuring the toxicity of sediment, pore water, or elutriate, because they are based on sensitive organisms that live in the water column but frequently come in close contact with sediment (e.g., EC, 1990a; 1992c; d; e). Guidance documents describing suitable procedures for collecting and manipulating sediment samples and for spiking clean sediment with chemicals in preparation for reference toxicity tests, have been prepared and are also available (EC, 1994; 1995).

The biological test method detailed in this report provides a standardized procedure for conducting a chronic test for sediment toxicity. It uses larvae of freshwater midges (*Chironomus tentans* or *C. riparius*), and measurements of survival and weight attained by the larvae at test end. The widespread distribution and common occurrence of *Chironomus* sp. in association with freshwater sediment, together with their ecological importance, ease of culturing and testing, rapid growth, short life cycle, sensitivity to contaminated sediment, and extensive use in sediment toxicity tests, led to the selection of this test method for standardization by Environment Canada. This method includes two test options: (1) an intermittent-renewal test in keeping with USEPA (1994a) and ASTM (1995a), which requires or recommends twice-daily renewal of the overlying water in test chambers and normally no aeration of the overlying water; and (2) a static toxicity test in which the overlying water is not renewed (except for



replacing losses due to evaporation) and is aerated continuously. Using either test option, the animals are fed ground tropical fishfood flakes, either as a daily ration of 6.0 mg dry solids per feed or four times only (nonconsecutive days) with 15.0 mg dry solids per feed.

Tests with samples of freshwater sediment and laboratory cultured larvae of freshwater midges (*Chironomus tentans* or *C. riparius*) have historically been carried out by Canadian, United States, and European investigators using a variety of culturing and test procedures. A partial summary of these procedures, their similarities and differences, is provided in Appendices C, D, and E. Notable draft, in-house, or published procedures for culturing *Chironomus* sp. and measuring their responses to sediment or other materials, which have influenced the preparation of this biological test method, include: Credland, 1973a; Townsend *et al.*, 1981; Nebeker *et al.*, 1984; McCahon and Pascoe, 1988; Ingersoll and Nelson, 1990; ASTM, 1991a; 1994; 1995a; USEPA, 1991a; 1994a; Bedard *et al.*, 1992; Hoke, 1992; NWRI, 1992; Ankley *et al.*, 1993; and Call *et al.*, 1993a. Of particular importance and influence were the United States Environmental Protection Agency's (USEPA) new methods for measuring the toxicity of sediment-associated contaminants using freshwater invertebrates (USEPA, 1994a), which were kindly provided to us in draft form<sup>1</sup>. These (USEPA, 1994a)

methods, which have been largely incorporated as part of the ASTM (1995a) compilation of standard test methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants<sup>2</sup>, include a 10-day test for sediment effects on survival and growth of larval *Chironomus tentans*. The biological test method herein relies heavily on the specific procedures for culturing and testing *C. tentans* detailed in USEPA (1994a); as well as on the intra- and interlaboratory studies using *C. riparius* and static or static-renewal test options, which were undertaken or coordinated by Environment Canada researchers to develop and validate the test method described in this report (Milani *et al.*, 1996).

Universal procedures for preparing for and conducting sediment toxicity tests using either *C. tentans* or *C. riparius* are described in this report. Also presented are specific conditions and procedures required or recommended when using the test for evaluating different types of substances or materials (e.g., samples of field-collected sediment or particulate waste, or samples of one or more chemicals or chemical substances experimentally mixed into or placed in contact with natural or formulated sediment). The flowchart in Figure 1 gives a general picture of the universal topics covered herein, and lists topics specific to testing samples of field-collected sediment, similar particulate material (e.g., sludge, drilling mud, or dredged material), or

<sup>1</sup> USEPA (1994a) was prepared by members of the United States Freshwater Sediment Toxicity Assessment Committee, and reflects a consensus opinion of U.S. and Canadian researchers actively engaged in sediment toxicity tests including those using larvae of freshwater midges (*C. tentans* or *C. riparius*) (Burton and Ingersoll, 1994; Ingersoll *et al.*, 1995;

Burton *et al.*, 1996).

<sup>2</sup> ASTM (1995a) includes a variety of procedures for culturing and testing both *C. riparius* and *C. tentans*, as well as guidelines for conducting sediment toxicity tests with several other species of freshwater invertebrates.

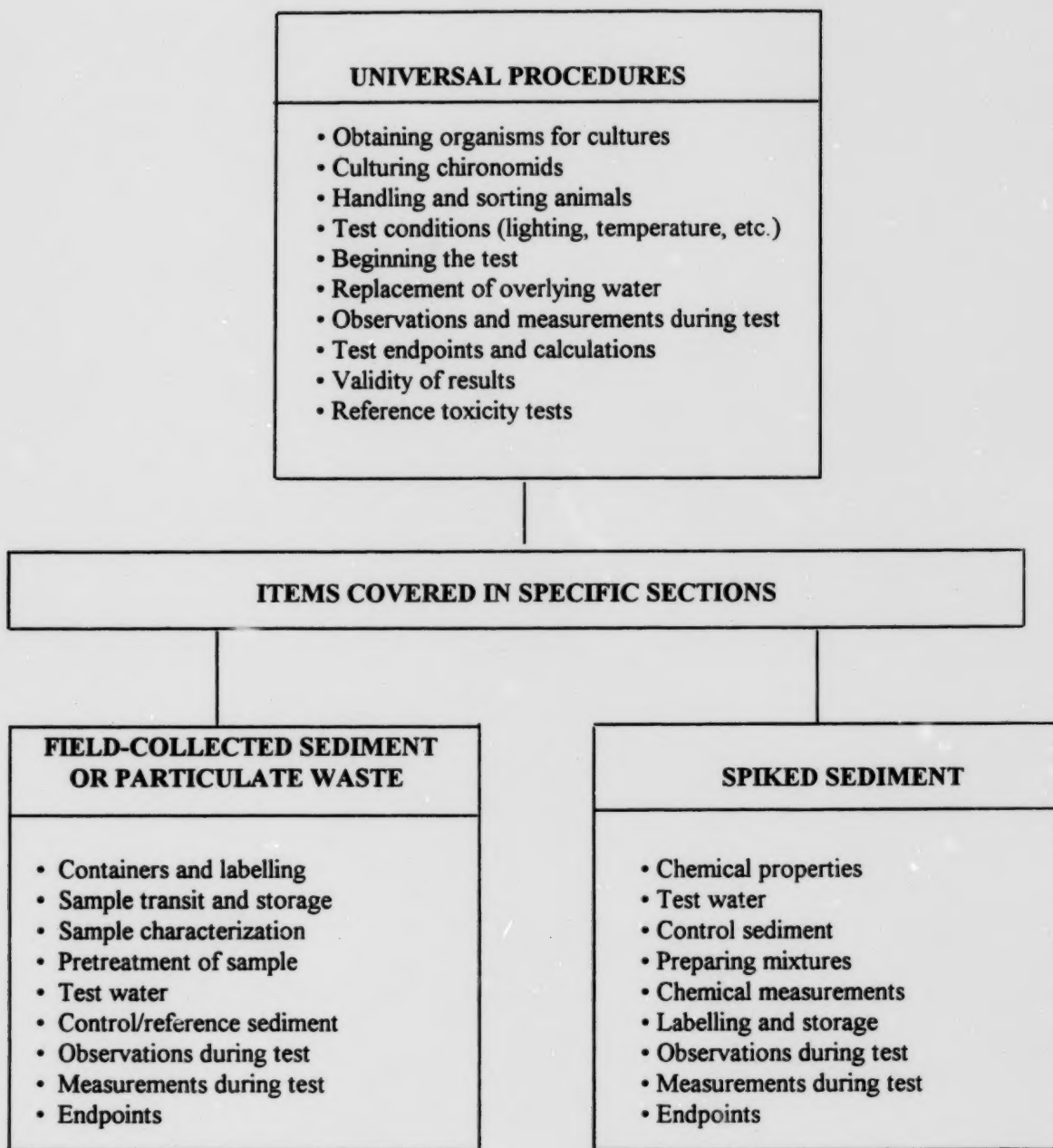


**Figure 1** Considerations for Preparing and Performing Toxicity Tests Using *Chironomus* sp. and Various Types of Test Materials or Substances

sediment spiked experimentally with chemical(s) or chemical substance(s), contaminated sediment, or particulate waste.

This biological test method has been developed following a review of variations in specific culturing and test procedures indicated in existing Canadian, United States, and European methodology documents<sup>3</sup> that describe how to prepare for and conduct sediment toxicity tests using larvae of the freshwater midges *C. tentans* or *C. riparius*. A summary of existing or past procedural variations for culturing these species is found in Appendix C. A summary of variations in existing or past procedures for conducting *survival-and-growth* tests for sediment toxicity using *Chironomus* sp. is found in Appendix D. Appendix E provides a summary of interlaboratory variations in conditions and procedures for undertaking *water only* reference toxicity tests with *Chironomus* sp.

The biological endpoints for this test method are percent survival and mean dry weight (as an indication of growth) at the end of the 10-day test. The test method is intended for use in evaluating the toxicity of samples of:

- (1) field-collected freshwater sediment;
- (2) industrial or municipal sludge and similar particulate wastes that might

affect the freshwater environment;  
and

- (3) mixtures of one or more chemicals or chemical substances within or overlying freshwater sediment.

A 14-day test method for toxic effects of these materials on survival and growth of the freshwater amphipod *Hyalella azteca* has also been developed by Environment Canada (1997a), and may be used in conjunction with, or as an alternative to, this test.

In formulating these procedures, an attempt has been made to balance scientific, practical, and cost considerations, and to ensure that the results will be sufficiently precise for most situations in which they will be applied. It is assumed that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions that might be required in a regulatory protocol are not provided, although this report is intended as a guidance document useful for that and other applications.

For guidance on the implementation of this and other biological test methods, and on the interpretation and application of the endpoint data, the reader should consult Environment Canada (1997c).

## 1.2 Identification, Distribution, and Life History

*Chironomus tentans* and *C. riparius* (Diptera: Chironomidae) are widely distributed (holarctic) nonbiting flies, commonly referred to as *midges*. The distinguishing features of *C. tentans* are described and illustrated in Appendix F, together with information on the distribution

<sup>3</sup> Documents used in preparing listings of procedural specific variations (see Appendices C, D, and E) include published "how-to" references, unpublished Standard Operating Procedures of governmental testing facilities, and draft reports. Citations of source documents are listed in these appendices by originating agency, rather than by author(s), although the authors and formal citations are identified in the appendices.

and life history of this species. Similar information for *C. riparius* is given in Appendix G.

These insects have four life stages: egg, larva, pupa, and adult. After mating, adult females of each species oviposit a single transparent, gelatinous egg mass directly into the surficial water of lakes, ponds, streams, or rivers.<sup>4</sup> Numbers of embryos per egg mass can average 2300 for *C. tentans*<sup>5</sup> (ASTM, 1995a), and 150 to 450 for *C. riparius* (Ingersoll *et al.*, 1990). In water at a temperature of between 19 and 23°C, eggs of each species hatch within 2 to 4 days (ASTM, 1995a). On the second or third day after hatching, the larvae burrow into the sediment and begin to construct tubes (or cases) which are open at each end. Larvae of each species are detrital-feeding and infaunal. Larval growth occurs in four instars of about 4 to 7 days each, depending on temperature. The four larval stages are followed by a pupal stage which lasts about 3 days, and then emergence to a terrestrial adult stage. The adult stage lasts for 3 to 5 days, during which the adults mate in flight and the females oviposit their egg masses (Townsend *et al.*, 1981; ASTM, 1991a; 1994; Call *et al.*, 1993a; USEPA, 1994a; ASTM, 1995a).

<sup>4</sup> Occasionally, female *C. tentans* reared under laboratory conditions will oviposit a smaller, second egg mass within 24 h. Its viability, however, is in question (K. Liber, Univ. Wisconsin, Superior, WI, personal communication, 1994).

<sup>5</sup> Average numbers at some laboratories might at times be only ~800 to 1500 (K. Liber, Univ. Wisconsin, Superior, WI, personal communication, 1994).

Midge larvae are important in the diet of fish and waterfowl (Townsend *et al.*, 1981; ASTM, 1995a). Most larvae of each species are found in the top few centimetres of sediment. Larvae frequently extend their anterior ends out of their tubes, and feed on the sediment surface. Very few *C. tentans* have been found below 40 cm depth; however, *C. riparius* can reportedly live to depths of 1 m. Larvae of *C. tentans* have been found in sediment with particles ranging from <0.15 mm to 2.0 mm. Larvae of *C. riparius* have been found in various substrates, including silt, limestone bottoms, and gravel (ASTM, 1991a; 1994; 1995a).

Larval *C. tentans* have been recovered from British Columbia lakes with temperatures ranging from 0 to 24°C, dissolved oxygen of 0.2 to 8.2 mg/L, pH of 8.0 to 9.2, conductivity of 481 to 4136 µmhos/cm, and sediment organic carbon of 1.9 to 15.5%; but were absent if the concentration of hydrogen sulphide in the overlying water exceeded 0.3 mg/L (Topping, 1971). Other studies have reported a temperature range of 0 to 35°C and a pH range of 7 to 10 for larvae of this species (ASTM, 1995a). *C. riparius* larvae reportedly occur in the field at temperatures ranging from 0 to 33°C, dissolved oxygen concentrations as low as 1 mg/L, and pH ranges of 5 to 9 (ASTM, 1995a). Further details concerning the life history and distribution of each species is given in Appendices F (*C. tentans*) and G (*C. riparius*).

### 1.3 Historical Use in Toxicity Tests

Burton (1991) provides an excellent review of the various methods used historically for measuring the acute or chronic toxicity of freshwater sediments, including toxicity tests



using chironomid larvae and other aquatic insects. Wentzel *et al.* (1977a) first reported a chronic (17-day) test for effects of contaminated sediment on survival and growth of midge larvae, using second instar *C. tentans*. Nebeker *et al.* (1984) described procedures for studying the effects of samples of contaminated sediment on both survival and growth of second instar *C. tentans* (15-day test) and on adult emergence of these organisms (25-day test). Results for each of these procedures have also been reported (Nebeker *et al.*, 1988). Ingersoll and Nelson (1990) published procedures and results for sediment toxicity tests of 13 days (for growth and survival) or 29 days (for adult emergence) duration, using first instar *C. riparius*. Benoit and Ankley (1993) described methods for partial life-cycle tests (first instar to emerging adults) with *C. tentans*, using samples of sediment. Ingersoll *et al.* (1995) reviewed methods and applications for sediment toxicity tests using midge larvae.

Reports of 10- to 14-day *survival-and-growth* tests, using samples of field-collected sediment and larval *C. tentans* or *C. riparius*, are now commonplace (e.g., Giesy *et al.*, 1990; Othoudt *et al.*, 1991; Ankley *et al.*, 1993; Becker and Bigham, 1993; Call *et al.*, 1993b; Sibley *et al.*, 1993; Kemble *et al.*, 1994; Becker *et al.*, 1995; Day *et al.*, 1995a; b; Reynoldson *et al.*, 1995; Bedard and Ali, 1996; Burton *et al.*, 1996; Milani *et al.*, 1996). Tests with midge larvae, using samples of field-collected sediment, have sometimes restricted the biological endpoint to survival (e.g., Schubauer-Berigan and Ankley, 1991; West *et al.*, 1993), and test duration to as short as 4 days. Researchers have sometimes extracted pore water from samples of field-collected sediment, and/or

prepared aqueous elutriates of sediment samples, then tested the effects of these extracts on growth and/or survival of midge larvae (Giesy *et al.*, 1990; Sibley *et al.*, 1993). Occasionally, larval toxicity tests with samples of contaminated sediment diluted with clean control sediment have been reported (Giesy *et al.*, 1990). The USEPA and the United States Army Corps of Engineers (USEPA/USACE, 1994) have recommended that *C. tentans* and/or *C. riparius* be used for evaluating the acute or chronic toxicity of dredged material proposed for discharge in inland waters.

Numerous *water only* acute lethality tests with various inorganic or organic chemicals have been conducted using chironomid larvae (*C. tentans* or *C. riparius*; first to fourth instars, depending on test) and, in certain studies, other species of freshwater invertebrates (e.g., Gauss *et al.*, 1985; Green *et al.*, 1985; 1986; Williams *et al.*, 1985; 1986a; b; Powlesland and George, 1986; Brown and Pascoe, 1988; Ingersoll *et al.*, 1990). The duration of these tests has been typically 2 to 4 days. More prolonged (10- to 30-day) *water only* tests with chemicals and midge larvae have also been performed, with survival, survival and growth, or adult emergence as the biological endpoint(s) measured (e.g., Powlesland and George, 1986; Pascoe *et al.*, 1989; Ingersoll *et al.*, 1990; Taylor *et al.*, 1991a; b; Suedel *et al.*, 1993a; West *et al.*, 1993; Hoke *et al.*, 1995; Schubauer-Berigan *et al.*, 1995). Substrate was sometimes available for the midge larvae (Pascoe *et al.*, 1989; Taylor *et al.*, 1991a; b), although in most of these *water only* tests no substrate was added.

Tests of sediment or water quality using midge larvae have included a number of

other approaches and applications. Several studies have reported spiking clean control sediment using inorganic or organic contaminants, with subsequent tests for toxicity of the mixture using larval *C. tentans* or *C. riparius* and survival, survival and growth, or adult emergence as the endpoints (Cairns *et al.*, 1984; Pittinger *et al.*, 1989; Lydy *et al.*, 1990; Douglas *et al.*, 1993; Harrahy and Clements, 1993; Suedel *et al.*, 1993a; Hoke *et al.*, 1995; Whiteman *et al.*, 1996). Quantitative effects of contaminants in sediment or water on the behaviour of chironomid larvae (e.g., percent of larvae avoiding contaminated sediment; timed percent of larvae burying in sediment and constructing tubes) or adults (e.g., numbers of egg cases deposited in a range of concentrations of chemical in water) have sometimes been measured as biological endpoints (Wentzel *et al.*, 1977b; 1978; Pascoe *et al.*, 1991; Suedel *et al.*, 1993a). *In-situ* tests for toxicity of sediment or overlying water have been undertaken using caged larvae of *C. tentans* (Nebeker *et al.*, 1984) or *C. riparius* (Ormerod *et al.*, 1987). Certain *water only* or *whole sediment* tests with midge larvae have measured the bioaccumulation and elimination of specific contaminants of concern, either at the end of a toxicity test or as a separate assay (Dodge and Theis, 1979; Mosher *et al.*, 1982; Nebeker *et al.*, 1984; Lydy *et al.*, 1990; 1992; Taylor *et al.*, 1991b; Harrahy and Clements, 1993).

Results of laboratory sediment toxicity tests using larval *C. tentans* or *C. riparius* have been examined to assess their value in identifying sites where natural populations of benthic organisms are affected by toxic sediment contaminants (Wentzel *et al.*, 1977a; b; Giesy *et al.*, 1988; 1990; Becker

and Bigham, 1993; Burton and Ingersoll, 1994; USEPA, 1994a; Day *et al.*, 1995a). Such *field-validation* studies typically integrate the findings of the laboratory toxicity tests with concurrent chemical analyses of sediment samples and field surveys of the diversity and abundance of benthic communities, using a Sediment Quality Triad (Chapman *et al.*, 1986; 1987; 1991) or similar approach. Studies to date indicate that sediment toxicity tests using midge larvae can provide reliable evidence of biologically adverse contamination of sediment in the field. For instance, Wentzel *et al.* (1977a; b; 1978) demonstrated a correlation between the abundance of larval *C. tentans* at sites where sediments were contaminated with metals, and sublethal responses of *C. tentans* to these sediments in the laboratory. Studies by Giesy *et al.* (1988; 1990) showed that sites where larval *C. tentans* were eliminated from contaminated Detroit River sediments were correlated with laboratory findings of 30 to 50% growth reduction of *C. tentans* larvae held in samples of sediment from these same sites.

#### 1.4 Laboratory Tolerance and Relative Sensitivity

Midge larvae are robust, and can withstand a wide range of laboratory conditions. Townsend *et al.* (1981) summarized the laboratory tolerance of *C. tentans* to various temperature, dissolved oxygen, pH, and lighting conditions. Temperatures ranging from 15 to 28°C were all compatible with a high (≥95%) percent hatch of embryos; more extreme temperatures reduced hatching success. Development of larval *C. tentans* has been demonstrated to be arrested at temperatures below 8°C. Hatching success

of this species was normal when dissolved oxygen concentrations ranged from 1 to 12 mg/L, whereas embryogenesis was terminated at  $<1$  mg  $O_2$ /L. Concentrations of dissolved oxygen  $\leq 3$  mg/L caused larval *C. tentans* to migrate into the water column and begin constructing tubes out of the sediment.<sup>6</sup> Development of *C. tentans* was described as normal at pH values of 5.5 or 6.5, whereas a pH of 4.5 caused 60% reduction in adult emergence and a pH of 3.5 caused 100% mortality of first instars. Short daylight hours (8L:16D) have been shown to arrest larval development at the fourth instar; development was normal with photoperiods of 16L:8D up to and including continuous (24L:0D) light (Townsend *et al.*, 1981).

Larval *C. tentans* and *C. riparius* can tolerate a wide range of substrates. Ingersoll and Nelson (1990) reported that *C. riparius* could be reared in the laboratory in clean sediment with particle sizes ranging from  $>90\%$  silt and clay to 100% sand, without detrimental effects on survival or growth. Bedard (1989) noted that growth of larval *C. tentans* was enhanced in the laboratory by coarser substrates with  $>80\%$  sand. Results for 10-day tests with larval *C. tentans* held in 50 samples of uncontaminated Great Lakes sediment having particle sizes ranging from 95% clay to 100% sand and an organic carbon content from 0.3 to 8.1% suggested that growth might have been slightly influenced by grain size, with some improvement in growth in the coarser sediments (Ankley *et al.*, 1994). Ten-day tests using larval *C. riparius* held in samples

of uncontaminated sediment with an organic carbon content ranging from 0.6 to 8.8% indicated that, within this range, the concentration of organic carbon did not influence survival or growth to a significant extent (Milani *et al.*, 1996). In life cycle studies with laboratory formulated sediments, Suedel *et al.* (1993b) found that *C. tentans* was tolerant of all particle size regimes examined (i.e., 0 to 100% sand, 0 to 100% silt, and 0 to 60% clay), but was intolerant of formulated control sediment with low ( $\leq 1.0\%$ ) organic content. In 10-day studies with formulated control sediment, Suedel and Rodgers (1994a) found that larval *C. tentans* survived well if the organic content was 3 to 5%, whereas organics of 0.5% caused a significant reduction in percent survival and only 33% of larvae survived in formulated control sediment with 0.0% organics. Similar findings are reported in Suedel and Rodgers (1994b), together with results showing adequate ( $\geq 80\%$ ) survival for fed *C. tentans* when held for 10 days in formulated sediment with 2.5% organics and particle sizes ranging from 100% sand to 100% silt. Suedel and Rodgers (1994b) also found poor (10 to 50%) survival rates for *C. tentans* fed daily during a 10-day exposure to several samples of field-collected sediment from "relatively pristine areas" which were low (0.12 to 0.19%) in organic content.

The relative sensitivity of various life stages of *C. tentans* or *C. riparius* to aquatic contaminants has been compared in a number of studies. In water only or spiked sediment tests, eggs were generally very resistant to toxicants, compared to recently hatched larvae (Gauss *et al.*, 1985; Williams *et al.*, 1987). In acute or chronic tests comparing the sensitivity of various instars of midge

<sup>6</sup> Larval *C. tentans* have been observed to behave normally at mean DO levels  $\sim 3$  mg/L, even when those levels were maintained for several days (K. Liber, Univ. Wisconsin, Superior, WI, personal communication, 1994).



larvae, results have indicated that tolerance increases progressively with instar number (Gauss *et al.*, 1985; Powlesland and George, 1986; Williams *et al.*, 1986b; Pascoe *et al.*, 1989). For instance, acute *water only* tests with copper and *C. tentans* found that first instar larvae were 12 to 27 times more sensitive than fourth instars (Gauss *et al.*, 1985), and similar tests with cadmium and *C. riparius* showed that first instars were 127 times more sensitive than second instars and 950 times more sensitive than fourth instars (Williams *et al.*, 1986b). Acute *water only* tests with *C. tentans* and nickel showed that first instars were twice as sensitive as second instars. Results for chronic toxicity tests with *C. riparius* and cadmium indicated that pupae were as tolerant as fourth instars (Pascoe *et al.*, 1989). In life cycle studies with *C. riparius* and lindane, Taylor *et al.* (1993) found that pupation and adult emergence were the most sensitive endpoints; however, differences in larval weight were not measured in this test, and the authors speculated that growth might be more sensitive than the integrated responses of life cycle tests.

Results for both *water only* and *whole sediment* tests with larval *C. tentans* or *C. riparius* demonstrate convincingly that growth is a sensitive endpoint, and is often appreciably more sensitive than an endpoint based on acute or chronic survival rates. For example, Powlesland and George (1986) compared results for acute (48 h) and chronic (30 d) *water only* tests with nickel and *C. riparius* larvae. Results showed that 30-day growth was 73 times more sensitive as an endpoint than acute (48-h) survival using first instars. In a series of 10-day *survival-and-growth* tests with second instar *C. riparius* and various chemicals in water,

Taylor *et al.* (1991b) found that chronic values based on growth were 6 to 100 times more sensitive (lower) than those based on survival. Numerous 10-day *survival-and-growth* tests with larval *C. tentans* and samples of contaminated sediment have also found that larval growth is a sensitive, biologically significant endpoint, and that survival alone does not adequately describe the potential long-term effects of sediment toxicity (Call *et al.*, 1993b; USEPA, 1994a). For instance, 10-day tests with larval *C. tentans* exposed to samples of Great Lakes sediment showed growth retardation for several samples, although survival was not adversely affected (Call *et al.*, 1993b). A series of comparative growth and life-cycle tests with *C. tentans* by Liber *et al.* (1996) demonstrated that a 35 to 50% growth retardation during 10-day larval tests resulted in similar reductions in adult emergence success.

Several studies have examined the relative sensitivity of midge larvae to specific chemicals, pore water, or contaminated sediment. In comparative *water only* tests using a number of species of freshwater invertebrates including amphipods, the sensitivity of larval *C. riparius* was sometimes ranked low (Green *et al.*, 1985; Williams *et al.*, 1985) or intermediate (Green *et al.*, 1986; Williams *et al.*, 1986a; Brown and Pascoe, 1988). However, these comparative tests used third or fourth instars of midge larvae. Comparative acute or chronic *water only* tests using first or second instars of midge larvae have shown that early instar *C. tentans* or *C. riparius* are often as sensitive or more so than daphnids or amphipods (Cairns *et al.*, 1984; Ingersoll *et al.*, 1990; Schubauer-Bergigan and Ankley, 1991; Taylor *et al.*, 1991a; Suedel *et al.*,

1993a; Phipps *et al.*, 1995). Chronic ( $\geq 10$  d) survival or *survival-and-growth* tests with samples of contaminated sediment from field collections, or with sediment spiked with chemical, have also shown that first or second instars of *C. tentans* or *C. riparius* can be as or more sensitive than daphnids (*Daphnia magna*) or amphipods (*Hyaella*

*azteca*), depending on the sediment samples compared (Ingersoll and Nelson, 1990; Othoudt *et al.*, 1991; West *et al.*, 1993; USEPA, 1994a). No reports of the relative sensitivity of larval *C. tentans* versus *C. riparius* to contaminated sediment or water have been found in the technical literature.

## Section 2

# Test Organisms

## 2.1 Species and Life Stage

*Chironomus tentans* Fabricius (Diptera: Chironomidae) or *Chironomus riparius* Meigen are the species of organisms that must be used for this test method. Species identification should be confirmed and documented by qualified personnel experienced in identifying chironomids, using the distinguishing taxonomic features described and illustrated in Appendices F (*C. tentans*) and G (*C. riparius*).

The sediment toxicity tests described herein must be started using either third instar *C. tentans* or first instar *C. riparius* (see Section 4.1, footnote 24). Organisms used in toxicity tests should be cultured in the laboratory at  $23 \pm 1^\circ\text{C}$  (see Section 2.3.5). Norberg-King (1994) found that third instar *C. tentans* had a mean head capsule width of 0.38 mm (range, 0.33 to 0.45 mm) and were 9 to 13 days post-hatch, at a temperature of  $23^\circ\text{C}$ . First instar *C. riparius* cultured at  $23^\circ\text{C}$  typically have a mean head capsule width of ~0.1 mm (range, 0.09 to 0.12 mm) and are normally 0 to 3 days post-hatch (Day *et al.*, 1994; K. Day, unpublished data).

## 2.2 Source and Acclimation

All *Chironomus* sp. used in a test must be derived from the same population. Sources of animals required to establish cultures (see Section 2.3) may be government or private laboratories which are culturing larval *C. tentans* or *C. riparius* for sediment toxicity

tests, or a commercial biological supplier<sup>7</sup>. Breeding stock can be acquired from the following Canadian sources:

### *Chironomus tentans*:

Aquatic Toxicity Section  
Standards Development Branch  
Ministry of Environment and Energy  
125 Resources Road  
Etobicoke, ON, M9P 3V6

Aquatic Toxicology Section  
Pacific Environmental Science Centre  
Environment Canada  
2645 Dollarton Highway  
North Vancouver, BC, V7H 1V2

### *Chironomus riparius*:

Toxicology Laboratory  
Environmental Quality Section  
Environmental Science Centre  
Environment Canada  
P.O. Box 23005  
Moncton, NB, E1A 6S8

<sup>7</sup> Investigators might be concerned with the effects of excessive inbreeding of laboratory cultures, or might wish to use progeny reproduced from organisms that occupied a particular locale. Accordingly, cultures may also be established using wild populations. If animals are obtained from a wild population, their taxonomy should be confirmed and they should be cultured through several generations and evaluated for sensitivity to reference toxicant(s) before the progeny are used in toxicity tests. Obtaining wild populations of organisms for testing should be avoided unless the ability of the wild population to cross-breed with existing laboratory populations has been demonstrated (USEPA, 1994a).

Persons wishing United States sources for *C. tentans* should refer to USEPA, 1994a (Table 10.1) for contacts.

Breeding stock for establishing cultures of *C. tentans* or *C. riparius* are best transported as recently laid egg masses. A suitable number (e.g.,  $\geq 5$ ) of egg masses should be transported to the laboratory using the source of water in which they were oviposited. Water used for transporting the egg masses should be well oxygenated (90 to 100% saturated) before shipment. Shipping containers should be insulated to minimize changes in water temperature during transit. Live organisms should be transported quickly to ensure prompt delivery (i.e., within 24 h). Excessive crowding of animals during shipment should be avoided to minimize stress and prevent oxygen deficiency in transit.

Upon arrival at the laboratory, egg masses may be held in the water used in transit while temperature adjustments are made, or they may be transferred to well-oxygenated culture water adjusted to the temperature of the water in the shipping container. Gradual exposure of organisms to culture water is recommended in instances where there is a marked difference in quality (e.g., hardness, pH, conductivity) from that to which they were previously acclimated. Guidance given in Section 4.1 for acclimating organisms to test water might also be followed here when transferring animals from another source to culture water.

Water temperature should be adjusted gradually to the temperature specified for culturing (23°C; Section 2.3), at a rate not exceeding 2°C/day (USEPA, 1994a). During this acclimation period, water used to

hold the breeding stock should be aerated gently. Other conditions during this interim holding period for acclimation of breeding stock to laboratory conditions should be as similar as possible to those used for maintaining cultures (Section 2.3).

## 2.3 Culturing

### 2.3.1 General

General guidance and recommendations for culturing *C. tentans* or *C. riparius* in preparation for sediment toxicity tests are provided here. In keeping with the premise "What might work well for one laboratory might not work as well for another laboratory" (USEPA, 1994a), explicit directions regarding many aspects of culturing, including the choice of culture chamber, number of organisms per chamber, water-renewal conditions, substrate for larvae, and food ration, are left to the discretion and experience of laboratory personnel, although guidance and recommendations are provided.

Performance-based criteria<sup>8</sup> are used to evaluate the suitability of the cultured organisms for tests, and the acceptability of the test results. To be suitable for use in tests, cultures must have low mortalities, and the cultured organisms must appear healthy and exhibit normal feeding and other behaviour. The acceptability of the culture should also be demonstrated by concurrent or ongoing tests using one or more reference

---

<sup>8</sup> Performance-based criteria include those related to the survival and condition of cultured animals intended for use in the test (Section 2.3.11); as well as the criteria that must be met by control organisms for a test to be valid (Section 4.2), and those related to the performance of groups of animals in reference toxicity tests (Section 4.8).

toxicants (Section 4.8). If a batch of organisms fails to meet these criteria, it should not be used for testing.

When initially setting up to perform sediment toxicity tests with *C. tentans* or *C. riparius*, it is the responsibility of the laboratory to demonstrate its ability to obtain consistent, precise results using one or more reference toxicants. For this purpose, intralaboratory precision, expressed as a coefficient of variation for the respective LC50 data, should be determined by performing five or more tests with different batches of test organisms from the same source, using the same reference toxicant and identical procedures and conditions for each test (see Section 4.8) (USEPA, 1994a). The laboratory should also confirm its test precision at this time by conducting five or more 10-day *survival-and-growth* tests using control sediment and different batches of test organisms (USEPA, 1994a). The conditions and procedures used to perform these initial tests with control sediment should be identical and according to Section 4.

When routinely performing sediment toxicity tests with midge larvae, reference toxicity tests should be conducted monthly with the laboratory's cultures, using the conditions and procedures outlined in Section 4.8. If this routine is not followed, the performance of individuals from the culture used to start a sediment toxicity test should be evaluated in a reference toxicity test conducted concurrently. Additionally, the performance of any cultures that have been recently established using new breeding stock (Section 2.2) should be checked with a reference toxicity test, and the results determined to be acceptable (see Sections

2.3.11 and 4.8) before these cultures are used to provide test organisms.

Cultures should be observed on a frequent and routine basis (e.g., daily or three times per week). The number of egg masses used to start each culture, dates of culture renewals, time to first emergence, estimated number of emerging adults, feeding schedule and rate, source of culture water and method/rate of renewal, water quality measurements, etc. should be documented (see Section 7 *Reporting Requirements*).

A summary of the various conditions and procedures that have been used by government laboratories for culturing *Chironomus* sp. is provided in Appendix C. These procedural specifics have presumably worked well in producing test organisms and, unless indicated otherwise in this report, provide useful guidance which may be applied here. A checklist of recommended conditions and procedures for culturing larval *C. tentans* or *C. riparius* to generate offspring for use in sediment toxicity tests is given in Table 1.

### 2.3.2 Facilities and Apparatus

*Chironomus* sp. must be cultured in a controlled-temperature laboratory facility. Equipment for temperature control (i.e., incubator, recirculating water bath, or constant-temperature room) must be adequate to maintain temperature within the required limits (Section 2.3.5). The culturing area must be isolated from any testing, sample-storage or sample-preparation areas, to avoid contamination from these sources. It must be designed and constructed to prevent contamination of cultures (e.g., elimination of copper or



**Table 1** Checklist of Recommended Conditions and Procedures for Culturing Larval *Chironomus* sp. for Use in Sediment Toxicity Tests

---

|                        |   |                                                                                                                                                                                                         |
|------------------------|---|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Source of chironomids  | - | existing government, private, or commercial culture; all animals in a test from the same source; species confirmed                                                                                      |
| Acclimation            | - | gradually ( $\leq 2^{\circ}\text{C}/\text{day}$ ) for temperature differences upon arrival                                                                                                              |
| Water source           | - | uncontaminated ground, surface, reconstituted, or, if necessary, dechlorinated municipal tap water                                                                                                      |
| Water quality          | - | temperature monitored daily; dissolved oxygen monitored at least weekly; pH, hardness and/or alkalinity, and ammonia measured during 24-h period preceding start of test                                |
| Culture chamber        | - | various (see Appendix C.2)                                                                                                                                                                              |
| No. organisms at start | - | various; recommend 150 to 200 first instar <i>C. tentans</i> , or 250 to 500 <i>C. riparius</i> , per 20-L culture chamber                                                                              |
| Water renewal          | - | intermittent-renewal or continuous-flow; $\geq 1$ volume addition/d recommended, 25 to 30%/week (minimum) unless water is recirculated through a suitable filter (see footnote 11)                      |
| Temperature            | - | $23 \pm 1^{\circ}\text{C}$ as daily average, and $23 \pm 0^{\circ}\text{C}$ as instantaneous                                                                                                            |
| Aeration/oxygen        | - | aerate gently; maintain dissolved oxygen at $\geq 80\%$ saturation                                                                                                                                      |
| Lighting               | - | 500 to 1000 lux adjacent to the water surface; overhead full-spectrum tubes (fluorescent or equivalent, with a broad-spectrum wavelength); photoperiod 16-h light : 8-h dark                            |
| Substrate              | - | silica sand or pulp from shredded, unbleached paper towels; recommended depth, 1 cm                                                                                                                     |
| Feeding                | - | ground tropical fish food flakes (e.g., Tetrafin <sup>TM</sup> or Nutrafin <sup>TM</sup> ); various quantities and rates allowed; recommend daily feeding of 300 mg dry weight per 20-L culture chamber |
| Age for test           | - | third instar <i>C. tentans</i> , or first instar <i>C. riparius</i>                                                                                                                                     |
| Health criteria        | - | discard any larvae that appear dead or inactive when gently prodded                                                                                                                                     |

---



galvanized piping or fixtures that could drip metal-contaminated condensates). The air supply to this area should be designed and operated to prevent entry or recirculation of air from the testing facility or from other portions of the laboratory where contaminants are present.

Any equipment, containers, or accessories that might contact the organisms or water within the culturing facility must be clean, rinsed as appropriate, and made of nontoxic materials (e.g., glass, Teflon<sup>TM</sup>, type 316 stainless steel, nylon, Nalgene<sup>TM</sup>, porcelain, polyethylene, polypropylene, fibreglass). Toxic materials including copper, zinc, brass, galvanized metal, lead, and natural rubber must not come in contact with this apparatus and equipment or the culture water. Online compressed air to the culturing facility should be filtered as necessary to ensure that it is free of oil and fumes.

### 2.3.3 Lighting

Overhead full-spectrum lights (fluorescent or equivalent) should illuminate the cultures. The photoperiod should be regulated at 16-h light and 8-h dark, and the light intensity adjacent to the water surface in the culture chambers should range within 500 to 1000 lux (USEPA, 1994a).

### 2.3.4 Culture Water

Sources of water for culturing *C. tentans* or *C. riparius* may be an uncontaminated supply of groundwater, surface water, or reconstituted water. Culture water may also be prepared by diluting natural water with a high purity distilled or deionized water until a desired hardness is achieved. Acceptable water must allow satisfactory survival, growth, and development of the organisms.

For certain site-specific investigations, the experimental design might require use of water taken from the site where sediment is collected. If this or other surface water is used, it should be filtered through a fine-mesh net (e.g., 30  $\mu\text{m}$ ) to remove potential predators or competitors. Water that might be contaminated with pathogens may be sterilized by passing it through an ultraviolet sterilizer.

In general, dechlorinated water is not recommended for use as culture or test water since its quality is often variable and it could contain unacceptably high concentrations of chlorine, chloramines, fluoride, copper, lead, zinc, or other contaminants.

Notwithstanding, certain laboratories routinely use dechlorinated municipal water for culturing *Chironomus* sp. and as test water, with no apparent problems. If municipal drinking water is used, effective dechlorination<sup>9</sup> must remove any harmful concentration of residual chlorine or chloramines.<sup>10</sup>

<sup>9</sup> Vigorous aeration of the water can be applied to strip out a portion of any residual chlorine gas. This could be followed by use of activated carbon (bone charcoal) filters and perhaps subsequent ultraviolet radiation (Armstrong and Scott, 1974) for removing most of the residual chloramine and other chlorinated organic compounds. Aging the water in an aerated holding tank for 1 or 2 days might be of further benefit.

<sup>10</sup> The target value for total residual chlorine, recommended for the protection of freshwater aquatic life, is  $\leq 0.002$  mg/L (CCREM, 1987). Values greater than 0.002 mg/L might risk interaction of chlorine/chloramine toxicity with the contaminants being tested. The limit of detection for the analytical technique used to measure residual chlorine or chloramine in the treated supply of dechlorinated water must be low enough to assure that residual chlorine is  $\leq 0.002$  mg/L.

If reconstituted fresh water is used for culturing and/or testing *C. tentans* or *C. riparius*, the recipe developed by the United States Environmental Protection Agency (USEPA) laboratory in Cincinnati, OH might prove suitable. The following recipe, which provides reconstituted water with a hardness of 90 to 100 mg  $\text{CaCO}_3/\text{L}$ , is taken from USEPA (1994a). It has a higher proportion of chloride to sulphate compared to other recipes commonly used (e.g., USEPA, 1985a; b; 1991b; 1993; EC, 1992b); and this recipe has been found to be suitable for culturing and/or testing *Chironomus* sp. (USEPA, 1994a; ASTM, 1995a). To prepare 100 L of reconstituted fresh water, use the reagent grade chemicals (anhydrous salts) as follows (USEPA, 1994a):

1. Place about 75 L of high purity deionized water in a clean (see Section 3.1) container.
2. Add 5 g of calcium sulphate ( $\text{CaSO}_4$ ) and 5 g of calcium chloride ( $\text{CaCl}_2$ ) to a 2-L aliquot of deionized water and mix (e.g., on a stir plate) for 30 minutes or until the salts dissolve.
3. Add 3 g of magnesium sulphate ( $\text{MgSO}_4$ ), 9.6 g sodium bicarbonate ( $\text{NaHCO}_3$ ), and 0.4 g potassium chloride (KCl) to a second 2-L aliquot of deionized water and mix on a stir plate for 30 minutes.
4. Pour the two, 2-L aliquots containing the dissolved salts into the 75 L of deionized water and fill the carboy to 100 L with deionized water.
5. Aerate the mixture for at least 24 h before use.

6. The water quality of the reconstituted water should be approximately the following: hardness, 90 to 100 mg/L as calcium carbonate ( $\text{CaCO}_3$ ); alkalinity, 50 to 70 mg/L as  $\text{CaCO}_3$ ; conductivity, 330 to 360  $\mu\text{S}/\text{cm}$ ; and pH, 7.8 to 8.2.

Reconstituted water should be aerated before use to adjust its dissolved oxygen to an acceptable range (see Section 2.3.6) and to stabilize pH. Conductivity, pH, hardness, dissolved oxygen, and alkalinity should be measured in each batch of reconstituted water (USEPA, 1994a). The USEPA (1991b) recommends using a batch of reconstituted water for two weeks but no longer than four weeks.

The characteristics of the water used within a laboratory for culturing *Chironomus* sp. should be reasonably uniform, in order to improve the likelihood of intralaboratory culturing success and to minimize variations in condition and development of cultured organisms. According to USEPA (1994a), a natural water is considered to be of uniform quality if monthly ranges of the hardness, alkalinity, and specific conductance are less than 10% of their respective averages, and if the monthly range of pH is less than 0.4.

The quality of water in culture chambers should be monitored and recorded routinely. Water temperature should be measured daily, and dissolved oxygen measured at least weekly. Culture water hardness, alkalinity, pH, and ammonia should be measured as frequently as necessary to document water quality. It is recommended that these variables be measured at least quarterly, as well as on the day before the start of a test (USEPA, 1994a).

Water used for culturing *Chironomus* sp. should be analyzed as appropriate (e.g., quarterly or more frequently) for nitrite, suspended solids, total dissolved gases, metals, pesticides, and any other contaminants of concern. For each analytical method used, the detection limit should be appreciably (e.g., 3 to 10 times) below either (a) the concentration in the water, or (b) the lowest concentration that has been shown to adversely affect the survival, growth, or development of *Chironomus* sp. or other sensitive freshwater animals (EC, 1992c).

The water within culture chambers should be renewed routinely.<sup>11</sup> This may be accomplished manually, or automatically using suitable apparatus and techniques for continuous or intermittent renewal. A water-renewal rate equivalent to  $\geq 1$  volume addition/day has been recommended (USEPA, 1994a), although such a frequent rate of exchange is likely unnecessary. A volume addition of 25 to 30% per week (NWRI, 1992) is the minimum exchange allowable unless water is recirculated through commercial (aquarium-supply) filters.

### 2.3.5 Temperature

The temperature of the water in culture chambers containing *C. tentans* or *C. riparius* should be  $23 \pm 1^\circ\text{C}$ , as a daily average (Table 1). Additionally, the

instantaneous temperature of the culture water should be  $23 \pm 3^\circ\text{C}$ .<sup>12</sup>

### 2.3.6 Dissolved Oxygen

Water to be used for cultures should be aerated vigorously just before use, to ensure an adequate oxygen content and to prevent its supersaturation with gases. Dissolved oxygen (DO) should be measured at this time to confirm that a satisfactory value has been obtained (e.g., 90 to 100% saturation).

Cultures should be aerated gently or moderately (Appendix C.4) using filtered, oil-free compressed air. Air to cultures should be dispensed through disposable airline tubing and disposable glass or plastic pipettes or, for large volume cultures, aquarium supply airstones. To ensure that dissolved oxygen (DO) is adequate to sustain optimum survival and growth of organisms, it is recommended that DO in cultures be maintained at 80 to 100% saturation.

### 2.3.7 Culturing Substrate

Various types of substrate have been used successfully for culturing *C. tentans* or *C.*

<sup>11</sup> Continuous recirculation of culture water through commercial aquarium supply filters can also maintain good quality water within cultures (G.A. Burton, Jr., Wright State Univ., Dayton, OH, personal communication, 1994), and such apparatus may be used as an alternative or supplement to water renewal.

<sup>12</sup> Certain organizations favour a somewhat lower temperature (e.g.,  $20 \pm 1^\circ\text{C}$  as the daily average temperature and  $20 \pm 3^\circ\text{C}$  as the instantaneous temperature) for culturing midge larvae and conducting sediment toxicity tests using survival and growth as biological endpoints (see Appendices C and D). Midge larvae may be cultured and tested within this somewhat lower mean temperature range (i.e., 20 to 22°C), provided that (i) the range for culturing and testing a particular group of organisms is essentially the same; (ii) the daily average temperature does not differ by more than  $\pm 1^\circ\text{C}$ ; (iii) the instantaneous temperature does not differ by more than  $\pm 3^\circ\text{C}$ ; and (iv) any deviation from the temperature range recommended here is acknowledged and reported. The investigator should be aware that instar development and/or growth might be somewhat lower than that at 23°C.

*riparius* (see Appendix C.5); the choice is left to the discretion and experience of laboratory personnel. The use of either clean (washed) silica sand, or washed pulp from shredded, unbleached paper towels, is recommended as a suitable substrate for midge larvae (Ingersoll and Nelson, 1990; ASTM, 1991a; 1994; 1995a; Bedard *et al.*, 1992; NWRI, 1992; BBA/IVA, 1994; USEPA, 1994a). Whatman No. 1 filter paper also provides a suitable and standard source of pulp for use as culturing substrate (D. Pascoe, Univ. Wales College, Cardiff, U.K., personal communication, 1994). A depth of 1 cm of substrate in culture chambers is recommended.

### 2.3.8 Food and Feeding

Various types of food and feeding regimes have been used for culturing *Chironomus* sp. (see Appendix C.6). Success in culturing midge larvae has been achieved using a single ration diet of commercial tropical fish food flakes such as Tetrafin™ or Nutrafin™ (NWRI, 1992; OME, 1992; Call *et al.*, 1993a; Ankley *et al.*, 1993; USEPA, 1994a; Milani *et al.*, 1996).<sup>13</sup> The use of these or other commercial fish food flakes, e.g., TetraMin™, is recommended for culturing midge larvae and as food in the tests (Section 4.4). The choice of quantities of commercial fish food to be added to each culture chamber, and feeding frequency, is left to the discretion and experience of laboratory personnel. If cultures have been started in 20-L aquaria, with 150 to 200 first instar *C. tentans* or 250 to 500 first instar *C. riparius* per aquarium, daily feeding of larvae

in each aquarium using 300 mg (dry weight) of ground fish food flakes is recommended (Table 1). Laboratories using static-renewal systems and infrequent (i.e., <1 volume addition/d) water renewal might need to use a lower feeding rate specific to their system. Instructions for food preparation and storage are given in Section 4.4.

### 2.3.9 Handling Organisms

Midge larvae should be handled as little as possible. When handling is necessary, it should be done gently, carefully, and quickly to minimize stress to the animals. Individual larvae can be transferred between containers using a glass or clear plastic pipette with a polished end and an opening of about 5 to 6 mm in diameter. Transferred organisms should be released below the water surface. When handled, any animals that are dropped, injured, contact dry surfaces, or appear stressed must not be used for testing.

### 2.3.10 Establishing and Maintaining Cultures

There are various options for the type, size, and loading densities of culture chambers (see Appendix C.2), as well as the type of culture water used and its method of replacement (Appendix C.3), choice of culturing substrate (Appendix C.5), and feeding conditions during culturing (Appendix C.6). For this test method, such choices are left largely to the discretion and experience of laboratory personnel; however, the culturing procedures used must produce enough midge larvae to start the planned toxicity tests with sediment and/or reference toxicant(s). Additionally, the cultured organisms must meet specific performance criteria (see Sections 2.3.11, 4.2, and 4.8).

<sup>13</sup> Thorough grinding of the flakes is advised to avoid the development of a fungal mat on the sand substrate in the culture tanks (K. Liber, Univ. Wisconsin, Superior, WI, personal communication, 1994).



To start a new culture, a sufficient number (normally at least five) of recently laid egg masses, obtained from either an outside source (Section 2.2) or from another culture within the laboratory, should be held at 23°C in a glass or nontoxic plastic (Section 2.3.2) beaker or dish containing about 100 to 150 mL of culture water. The egg masses should be examined periodically to determine hatching success. Once the embryos have started hatching (in about 2 to 4 days after ovipositing of egg masses by adult midges), it is recommended that a small amount (e.g., 4 mg Tetrafin™ or Nutrafin™) of suspended food be added to the water. When most of the larvae have left the egg masses, an appropriate number (e.g., 150 to 200 *C. tentans* or 250 to 500 *C. riparius*; Table 1) of the first instar larvae should be transferred to the culture chamber. Routine observations and record-keeping (Section 2.3.1), monitoring of water quality (Section 2.3.4), and feeding (Section 2.3.8) should be carried out throughout the period that the culture is maintained.

Water in each culture chamber should be changed routinely (see Section 2.3.4). The rate of water exchange should be increased (or the density of larvae reduced) if animals appear stressed or if the water is cloudy (USEPA, 1994a). If water is replaced manually, care should be taken not to pour or siphon out the larvae when removing the water. A small mesh sieve or net can be used to catch any larvae that float out. The sides of the culture chamber can be wiped clean or brushed off during such water exchanges. Any water used for replacement must be adjusted to the culture temperature (23°C) and aerated vigorously to assure its saturation with oxygen, before it is introduced to the culture chamber.

At 23°C, adult emergence will begin at about two (*C. riparius*) or three (*C. tentans*) weeks after hatching. Emerged adults can be gently siphoned into a dry aspirator bottle (e.g., a 250-mL or 500-mL Erlenmeyer flask; USEPA, 1994a) daily. Short inhalations should be used to avoid injuring the adults. Sex ratio of the adults should be checked to ensure that a sufficient number (e.g., ≥50%) of males is available for mating and fertilization (USEPA, 1994a).

A chamber for mating and oviposition should be prepared. The USEPA (1994a) provides useful guidance on suitable apparatus. The choice of chamber(s) for mating adults and oviposition of egg masses is optional and left to the discretion and experience of the laboratory.

#### 2.3.11 Health Criteria

Cultures should be checked three times per week (e.g., Monday, Wednesday, Friday) as a minimum and preferably daily. Records should be kept which monitor each culture's performance (see Sections 2.3.1, 2.3.4, 4.2, and 4.8). Procedures used to maintain each culture should be evaluated routinely, and adjusted as necessary to maintain or restore the health of the culture. Larvae in cultures that appear dead or inactive when gently prodded must not be used for testing.

Reference toxicity tests should be conducted monthly with the culture(s) of midge larvae, using the conditions and procedures outlined in Section 4.8. Alternatively, a reference toxicity test should be performed in conjunction with the sediment toxicity test. Test-related criteria used to judge the health and sensitivity of the culture are given in Sections 4.2 and 4.8.

Biochemical measurements such as the lipid content of cultured midge larvae might provide useful information on the health of the cultures (USEPA, 1994a). Other health criteria for future development could include a minimal average dry weight of *Chironomus* sp. at a certain age/instar. Low weights might indicate that cultured organisms

experienced sub-optimal growth conditions. Ongoing records of these or other indices of the condition of cultures will likely prove useful, and are encouraged. No specific health criteria have been developed as yet with respect to physiological measurements, although they could be applied in the future.



### Section 3

## Test System

### 3.1 Facilities and Apparatus

Tests may be performed in a water bath, environmental chamber, or equivalent facility having acceptable temperature and lighting (see Section 3.2) control. The test facility must maintain the daily mean temperature of all sediment and water in test chambers at  $23 \pm 1^\circ\text{C}$  (see Section 4.2). The facility should be well ventilated to prevent exposure of personnel to harmful fumes, and isolated from physical disturbances or any contaminants that might affect the test organisms. The area used to manipulate sediment in preparation for tests should also be properly ventilated.

The test facility should be isolated from the area where organisms are cultured, to avoid potential contamination of cultures. Additionally, the test facility should be removed from places where samples are stored or prepared, to prevent the possibility of contamination of test chambers and their contents from these sources. The ventilation system should be designed, inspected, and operated to prevent air within the testing facility from contaminating culture facilities. Return air from sample handling and storage facilities or those where chemicals are processed or tested should not be circulated to the region of the laboratory where tests are conducted. Any construction materials that might contact the organisms, water, or test chambers within this facility must be nontoxic (see Section 2.3.2).

Compressed air used within the test facility for aerating water must be free of oil and

fumes. Oil-free air pumps should be used wherever possible. Any oil or particulate in the air supply should be removed by online filters, which are replaced as required to ensure their effectiveness.

Equipment and supplies that contact water, sediment, or test chambers should be chosen to minimize sorption of chemicals. Borosilicate glass, nylon, high-density polyethylene, high-density polystyrene, polycarbonate, fluorocarbon plastics, and type 316 stainless steel should be used whenever possible to minimize chemical sorption and leaching. The use of apparatus and supplies made of toxic substances (see Section 2.3.2) must be avoided.

The test method may be performed as either a daily-renewal test, or as a static test (see Section 4). If the daily-renewal test option is selected, apparatus used to deliver and renew overlying water in test chambers may be one of several designs<sup>14</sup> (e.g., Maki, 1977; Benoit *et al.*, 1993; Zumwalt *et al.*, 1994). A suitable apparatus would be one that enables the timed and intermittent renewal of the overlying water in each test chamber at a rate of two volume additions per day (USEPA, 1994a). The automated water-renewal system designed by Zumwalt *et al.* (1994) is recommended for this test, since it is inexpensive, easy to build and calibrate,

---

<sup>14</sup> The overlying water may also be replaced manually, by siphoning. Manual systems, however, are more labour intensive and generally result in more suspension of sediment compared to automated systems (USEPA, 1994a).

and delivers small volumes of water to test chambers with minimal variation. The USEPA (1994a) provides useful guidance on the design of this and other suitable systems for the automated renewal (at a rate of two volume additions per day) of overlying water.

If the static test option is chosen, or for daily-renewal tests requiring aeration of the overlying water (see Section 4.3), a supply of disposable glass pipets and aquarium supply airline tubing is required for delivery of a continuous (gentle) flow of air to each test chamber. Stainless-steel (rather than brass) gang valves are recommended for regulating air flow. For static tests (including those with reference toxicant), a supply of suitably-sized watchglasses or lids is needed for covering individual test chambers.

The test facility must have the basic instruments required to monitor the quality (e.g., temperature, pH, dissolved oxygen, conductivity) of the test water and pore water. Additionally, the laboratory should be equipped to facilitate prompt and accurate analysis, with acceptable limits of detection, of such variables as hardness, alkalinity, ammonia, and (in instances where dechlorinated municipal water is used as culture or test water) residual chlorine.

All test chambers, equipment, and supplies that might contact sediment or test water must be clean and rinsed with test water, deionized water, or distilled water<sup>15</sup> before its use. All nondisposable materials should be washed after use. The following cleaning procedure (USEPA, 1994a) is recommended.

1. Soak in tap water for 15 minutes, then scrub with detergent or clean in an automatic dishwasher.
2. Rinse twice with tap water.
3. Rinse carefully with fresh, dilute (10%, v:v<sup>15</sup>) nitric (HNO<sub>3</sub>) or hydrochloric acid (HCl) to remove scale, metals, and bases.
4. Rinse twice with deionized water.
5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy).
6. Rinse three times with high-quality deionized water.

Test chambers and apparatus that might contact sediment or test water should be thoroughly rinsed with test water, immediately before use in the test.

Before toxicity tests are undertaken in a new test facility, a minimum of five, 96-h *water only* reference toxicity tests, and a minimum of five, 10-day survival-and-growth tests with control sediment, should be undertaken to confirm that acceptable performance of larval *C. tentans* or *C. riparius* can be achieved using the new facility and the culturing and test conditions and procedures specified in this report (see Sections 2.3.1 and 4). Each test with reference toxicant or control sediment should be performed using a different batch of cultured organisms. Data

---

<sup>15</sup> To prepare a 10% solution of acid, carefully add 10 mL of concentrated acid to 90 mL of deionized water.

from these preliminary tests should be compared by calculating and appraising the magnitude of the coefficient of variation for the respective series of tests and endpoint values.

### 3.2 Lighting

All test chambers should receive full-spectrum (e.g., fluorescent or equivalent) illumination from directly overhead, at an intensity sufficient to provide 500 to 1000 lux adjacent to the surface of overlying water in test chambers. Illumination should be as uniform as possible for all test chambers. Photoperiod should be regulated at 16-h light and 8-h dark.

### 3.3 Test Chambers

High form glass beakers or glass jars, with a capacity of 300 mL and an inner diameter of ~7 cm, are recommended as test chambers (USEPA, 1994a; ASTM, 1995a).<sup>16</sup> Each beaker or jar must be cleaned thoroughly before and after use (Section 3.1), and rinsed well with test water immediately before use. Covers for test chambers are not normally required for daily-renewal tests, although they should be used for static tests and are

<sup>16</sup> The use of test chambers meeting these specifications is recommended to conform with USEPA (1994a) and ASTM (1995a). However, comparative studies using *C. riparius* held in this configuration of test chamber or a larger (1.8-L glass jar) test vessel showed little if any difference in test results (Bedard, 1996). An investigator may choose to use a different volume and diameter of beaker or glass jar as a test chamber, provided that (i) this deviation from the test chamber recommended here is noted in the report; (ii) the type, shape, and size of the test chamber used is also reported; and (iii) identical test chambers are used for all replicates and treatments in a test.

also recommended for daily-renewal tests if test sediments contain detectable volatile gases. Suitable covers include clean watch glasses, or glass or plastic lids.

### 3.4 Test and Control/Dilution Water

Depending on the test design and intent (see Sections 5 and 6), test water (i.e., water overlying sediment in the test) and control/dilution water (i.e., water used to prepare dilutions of test chemicals and as control water in *water only* exposures with reference toxicants) may be either an uncontaminated supply of natural fresh water or reconstituted water. The water supply used as test or control/dilution water is frequently the same as that used for culturing *Chironomus* sp. (see Section 2.3.4), although it may come from another source. For instance, the use of site water, or clean water adjusted to the hardness of water at a collection site, might prove a good choice (see Section 5.4). The quality of test water and that used as control/dilution water is extremely important; this water must have been demonstrated to allow acceptable survival and growth of test organisms in 10-day tests with control sediment (see Section 4.2) before it is used in toxicity tests. Guidance for preparing reconstituted fresh water is provided in Section 2.3.4.

Test and control/dilution water must be adjusted to the test temperature before use. The dissolved oxygen content of the water should be 90 to 100% of the air saturation value at this temperature. As necessary, the required volume of water should be aerated vigorously (oil-free compressed air passed through air stones) immediately before use, and its dissolved oxygen content checked to

confirm that 90 to 100% saturation has been achieved. The pH of the water should be measured and stable before use.

### 3.5 Control Sediment

Each sediment toxicity test must include an experimental control, with a minimum of five replicate beakers or jars containing control sediment. A control sediment is a sediment that is essentially free of any contaminants that could adversely affect the survival, growth, or behaviour of *C. tentans* or *C. riparius* during the test. The use of control sediment provides a measure of test acceptability (i.e., mean survival at test end must be  $\geq 70\%$ ), evidence of the health and behaviour of the test organisms, and a basis for interpreting data derived from the test sediments.

Control sediment may be either natural sediment taken from a collection site removed from known sources of contaminants and shown previously to enable

acceptable survival and growth of *Chironomus* sp. under the conditions of the test, or formulated sediment.<sup>17</sup> The selection of an appropriate control sediment depends on considerations such as the study design, physicochemical characteristics of the test sediment(s), and the availability of suitable *clean* sediment having the desired properties. There should also be evidence that the sediment provides consistent and acceptable biological endpoints using *C. tentans* or *C. riparius* and this test method. While many clean, natural sediments have been used as control sediment in toxicity tests with *Chironomus* sp., the use of formulated control sediment offers a more consistent, standardized approach and one which numerous researchers are now actively pursuing (Dwyer *et al.*, 1993; Suedel and Rodgers, 1994a; b; USEPA, 1994a; Suedel *et al.*, 1996). At present, it is premature to recommend a recipe for formulated control sediment that is suitable for a 10-day *survival-and-growth* test using midge larvae.

---

<sup>17</sup> Formulated sediment is also described as reconstituted, artificial, or synthetic sediment. It is typically prepared using sand, silt, clay, and nontoxic organic constituents obtained from commercial sources, and is hydrated with reconstituted or natural water. Formulated sediment can be prepared to match different natural sediments with respect to particle size distribution, organic carbon content, pH, cation exchange capacity, etc. (Suedel and Rodgers, 1994a; Milani *et al.*, 1996). Alternatively, one or more recipes can be used to prepare standardized control sediment(s) for routine use in sediment toxicity tests with *C. tentans* or *C. riparius* (Suedel *et al.*, 1996).



## Section 4

### Universal Test Procedures

General procedures and conditions herein apply to each of the described toxicity tests for samples of sediment, particulate waste, or chemical, and to reference toxicity tests. More specific procedures for conducting tests with field-collected samples of sediment or other similar particulate material (e.g., sludge, dewatered mine tailings, drilling mud residue) are provided in Section 5. Guidance and specific procedures for conducting tests with control or other sediment spiked experimentally with chemical(s), contaminated sediment, or particulate waste are given in Section 6.

All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. The summary checklist of recommended test conditions and procedures in Table 2 describes not only universal procedures but also those for testing specific types of test materials or substances.

Universal procedures are described herein for performing a 10-day sediment toxicity test, which includes the following two test options<sup>18</sup>:

<sup>18</sup> Similar results are apparently obtained by static and static-renewal tests, when performed according to the procedures defined herein. The performance of these two test options was compared in side-by-side tests using samples of field-collected or contaminant-spiked sediment (Milani *et al.*, 1996). Results for interlaboratory tests with *C. riparius* suggested that the static option was somewhat less variable than the static-renewal option. Interlaboratory coefficients of variation (CVs) for grand means (all laboratories) of the 10-day survival data for field-collected sediment

- (1) a daily-renewal test, where the overlying water is renewed at a rate of two volume additions per day, and is normally not aerated; and
- (2) a static test, where the overlying water is not renewed during the test, but is aerated.

Using either test option, the larvae are fed ground tropical fish food flakes, either as a daily ration of 6.0 mg dry solids per feed or four times only (nonconsecutive days) with 15.0 mg dry solids per feed (see Section 4.4). Biological endpoints measured in this test method are survival and dry weight at test end.

#### 4.1 Beginning the Test

Each test chamber (see Section 3.3) placed within the test facility must be clearly coded

ranged from 2.1 to 5.5% using the static system, and from 7.5 to 17.0% using the static-renewal system. CVs for grand means of the weight attained by larvae in 10-day tests with these same samples ranged from 2.6 to 4.4% using the static system, and from 29.2 to 56.2% using the static-renewal system.

When each laboratory tested a range of concentrations of copper-spiked sediment, the grand-mean CVs for survival ranged from 2.1 to 25.1% using the static system and from 2.6 to 56.3% using the static-renewal system; the CVs for weight were from 7.7 to 27.4% (static) and from 29.2 to 75.9% (static-renewal). Statistical comparisons of 10-day LC50s and IC25s for these multi-concentration tests did not distinguish a consistent difference in sensitivity using either system (Milani *et al.*, 1996).

**Table 2** Checklist of Recommended Conditions and Procedures for Conducting Sediment Toxicity Tests using *Chironomus* sp.

---

**Universal**

|                        |   |                                                                                                                                                                                                                                                          |
|------------------------|---|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Test type              | - | 10-day whole sediment toxicity test with two options: daily renewal of overlying water; or no renewal (static test)                                                                                                                                      |
| Water renewal          | - | if using daily-renewal option, overlying water is replaced at a rate of 2 volume additions/day; if using static option, no renewal of overlying water during test except for replacement for losses due to evaporation                                   |
| Test (overlying) water | - | culture water or other clean ground or surface water; site water; water adjusted to hardness of site water; reconstituted fresh water for higher degree of standardization; dissolved oxygen, 90 to 100% saturation when used as overlying water in test |
| Control sediment       | - | sample of clean sediment that is used to assess the performance of the test organisms and the acceptability of the test; either natural or formulated sediment                                                                                           |
| Test organisms         | - | third instar <i>C. tentans</i> , or first instar <i>C. riparius</i> ( $\leq 48$ h post-hatch) to start test; 10/test chamber                                                                                                                             |
| Test chamber           | - | 300-mL high form glass beaker or glass jar; $\sim 7$ cm inner diameter; normally uncovered if daily-renewal test and covered if static test                                                                                                              |
| Volume of wet sediment | - | 100 mL                                                                                                                                                                                                                                                   |
| Volume of test water   | - | 175 mL                                                                                                                                                                                                                                                   |
| Number of replicates   | - | recommend $\geq 5$ field replicates, each a discrete (i.e., different) sample from the same location; must be $\geq 5$ laboratory replicates for each field replicate                                                                                    |
| Temperature            | - | daily average, $23 \pm 1^\circ\text{C}$ ; instantaneous, $23 \pm 3^\circ\text{C}$                                                                                                                                                                        |
| Lighting               | - | overhead full-spectrum (fluorescent or equivalent); 500 to 1000 lux; 16-h light:8-h dark                                                                                                                                                                 |



|                                 |   |                                                                                                                                                                                                                                                                                                                                |
|---------------------------------|---|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Aeration                        | - | if using daily-renewal option, none unless dissolved oxygen in overlying water drops below 40% of saturation; if using static option, continuous and minimal (e.g., 2 to 3 bubbles/s, each test chamber)                                                                                                                       |
| Feeding                         | - | ground tropical fish food flakes (e.g., Tetrafin™ or Nutrafin™), fed daily or four times only (nonconsecutive days); 6.0 mg dry solids in a 1.5 mL suspension added daily to each test chamber if daily feeding; 15.0 mg dry solids in a 3.75 mL suspension added to each test chamber on each feeding, if fed four times only |
| Observations                    | - | optional: numbers of organisms in each chamber seen on sediment surface, and their behaviour (daily or less frequently)                                                                                                                                                                                                        |
| Measurements of overlying water | - | ≥ 3 times/week, each treatment, for DO and temperature; start and end of test, each treatment, for pH, conductivity, and ammonia; recommend hardness and/or alkalinity at start and end                                                                                                                                        |
| Endpoints                       | - | significantly lower survival and weight than in control or reference treatments (based on mean percent survival and mean dry weight, each treatment; 10-d LC50 for multi-concentration test, where appropriate; ICp for weight, where appropriate)                                                                             |
| Test validity                   | - | invalid if mean 10-day survival in control sediment <70%; invalid if individual mean dry weight for controls at test end is <0.6 mg ( <i>C. tentans</i> ) or <0.5 mg ( <i>C. riparius</i> )                                                                                                                                    |

#### Field-collected Sediment or Similar Particulate Material

|                       |   |                                                                                                                                                                                                                                           |
|-----------------------|---|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Transport and storage | - | if sample >7°C, cool to 7°C (ice or frozen gel packs); transport in dark at 1 to 7°C (preferably 4 ± 2°C); store in dark at 4 ± 2°C; test should start within two weeks and must start within six weeks                                   |
| Reference sediment    | - | one or more samples for tests with field-collected sediment; taken from sites presumed to be clean but in the general vicinity of sites where test sediments are collected (i.e., same body of water); frequently selected for use in the |

toxicity test because of its physicochemical similarity (e.g., particle size and/or organic carbon content) to the test sediments

- |                         |   |                                                                                                                                                                                                                                                                                                                                                                                                                |
|-------------------------|---|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Sample characterization | - | at least particle size analysis (percent sand, silt, and clay), total organic carbon, percent moisture, porewater pH, and porewater ammonia                                                                                                                                                                                                                                                                    |
| Preparation of sample   | - | only if necessary, remove debris and indigenous macro-organisms using forceps; homogenize sample (including any separated liquid) before use in test; if necessary, remove smaller macro-organisms by pressing through fine-mesh sieve (e.g., 0.25 to 0.5 mm), or pass through fine-mesh sieve using liquid that separated from sample during transit and storage and remix this liquid with the sieved sample |

### Spiked Sediment

- |                                       |   |                                                                                                                                                                                                                                                                                                                               |
|---------------------------------------|---|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Characterization of chemical(s) added | - | information required on stability, water solubility, vapour pressure, purity, and biodegradability should be known for chemicals spiked into control sediment                                                                                                                                                                 |
| Solvent                               | - | test water is the preferred solvent; if an organic solvent is used, the test must include a solvent control                                                                                                                                                                                                                   |
| Preparation of mixtures               | - | procedure depends on test design and test objectives; might include one or more chemical concentrations mixed in control or test sediment, or specific chemical concentrations added to the test water overlying control sediment; chemical/sediment mixtures may be prepared manually or by mechanical agitation as slurries |
| Concentration of chemical(s) added    | - | normally measure at beginning and end of test, in high, medium, and low strengths as a minimum                                                                                                                                                                                                                                |
| Test and dilution water               | - | use reconstituted water if a high degree of standardization is required                                                                                                                                                                                                                                                       |
-

or labelled to enable identification of the sample or its concentration. The date and time when the test is started must be recorded, either directly on the labels or on separate data sheets dedicated to the test. The test chambers should be positioned for ease while making observations and measurements. Treatments should be positioned randomly within the test facility (USEPA, 1994a).

The day that midge larvae are initially exposed to samples of test materials or substances is designated Day 0. On the day preceding the start of the test (i.e., Day -1), each sample or subsample of test sediment or similar particulate material, including control and reference sediment, should be mixed thoroughly<sup>19</sup> (see Sections 5.3 and 6.2) to provide a homogeneous mixture consistent in colour, texture, and water content. Quantitative measures of homogeneity might include particle size analysis, total organic carbon, percent moisture, and concentrations of specific chemicals.

Immediately following mixing, replicate 100-mL volumes of the sample should be transferred to the test chambers.<sup>20</sup> A

<sup>19</sup> Any liquid that has separated from the sample during transport and/or storage must be remixed within the sample.

<sup>20</sup> It is recommended that 100 mL of sediment and 175 mL of test water be added to each 300-mL capacity test chamber, a practice used by USEPA (1994a) and ASTM (1995a). These quantities provide a water:sediment ratio of 1.75:1, which is appreciably lower than the ratios of 4:1 or 5:1 used by certain investigators (see Appendix D). Some evidence (Bedard, 1996) suggests that the water:sediment ratio might not be critical when standardizing the test method, although this might not always prove to be true. An investigator may choose to use a different type

minimum of five laboratory replicates per treatment must be established<sup>21</sup> (see Sections 5.1 and 6.2). The sediment added to each chamber should be smoothed using a spatula or by tapping the chamber against the side of the hand. Test water (see Section 3.4) is then poured slowly down the side of the beaker or jar. To minimize the disruption of sediment as test water is added, a disc made of Teflon<sup>TM</sup>, polyethylene, or nylon sheeting, cut to fit the inside diameter of the test chamber, may be placed on the sediment surface before water is added<sup>22</sup> (EC, 1992a).

For tests with daily renewal of the water overlying sediment, a total volume of 175 mL of test water (or, depending on the test, a test solution) should be added to the test chamber at this time; this can be judged using a 275-mL mark inscribed on the chamber's side. For static tests, a somewhat lesser volume (e.g., 125 to 145 mL) of test water may be added initially, to provide

of test chamber (see footnote 16) and/or modify the water:sediment ratio of 1.75:1 recommended here, provided that: (i) for a given test, the volume of water and sediment added to each test chamber is identical; (ii) these volumes are noted in the report together with the resultant water:sediment ratio; and (iii) the report notes the deviation from the recommended test volumes of water and sediment, and the resultant ratio.

<sup>21</sup> USEPA (1994a) indicates a minimum requirement of four replicates per treatment, and recommends eight replicates per treatment for sediment toxicity tests.

<sup>22</sup> A length of nylon monofilament line (or nontoxic equivalent) could be attached to the disc, to enable its removal once the test water is added. Alternatively, the disc could be cut from a polyethylene bag in a keyhole configuration which provides a circle with an attached portion for removal. The disc should be rinsed with test water if reused to prepare replicates of a treatment. A separate disc should be used for each treatment.

room for any additional water added when test organisms are introduced.

If the daily-renewal test option is chosen, water overlying the sediment in each test chamber should be renewed on the day preceding the test (Day -1) as well as throughout the test, at a rate of two volume additions per day<sup>23</sup> (USEPA, 1994a ; Table 2). A replacement rate greater than two volume additions per day should be avoided to prevent unnecessary flushing and depletion of any contaminants that might leach from the sediment into the overlying water. The overlying water is normally not aerated and test chambers are not covered, using this test option. The use of an automated intermittent-renewal system such as the one designed by Benoit *et al.* (1993) and modified by Zumwalt *et al.* (1994), is recommended (see Section 3.1); this apparatus can be set up to renew the overlying water at 12-h intervals. If an

---

<sup>23</sup> In water-renewal tests with one to four volume additions of overlying water/d, water quality characteristics generally remain similar to the inflowing water. However, in sediment toxicity tests where overlying water is not renewed or is replaced less frequently than once per day, the quality of the overlying water (e.g., conductivity, alkalinity, hardness) can change markedly. For instance, concentrations of hardness, alkalinity, conductivity, or metabolic products (e.g., ammonia) in the overlying water might increase (Ingersoll and Nelson, 1990; Ankley *et al.*, 1993a; USEPA, 1994a). Such changes in water quality characteristics could influence the test results, and can be avoided by the routine (i.e., twice daily) replacement of overlying water throughout the test period. Regardless, certain researchers prefer the static test option, due to the lesser labour and equipment requirements and the possibility that a daily- (or twice-daily) renewal test might deplete toxicants from the sediment during the test and perhaps reduce its potential toxic effects (Milani *et al.*, 1996).

automated system is used, it should be calibrated before the test is started to verify its performance; flow rates through any two test chambers should not differ by more than 10% at any time during the test (USEPA, 1994a; ASTM, 1995a). If overlying water is renewed twice daily by siphoning, care should be taken to prevent disturbance of the sediment or accidental loss of any animals emerged from the sediment during this procedure. No more than 90% of the water should be siphoned and replaced, and the end of the siphon must not contact the sediment.

If the static test option is chosen, the overlying water in each test chamber should be aerated overnight before the test organisms are introduced, as well as throughout the test (see Section 4.3). Each beaker or jar should be kept covered (watchglass or plastic lid) during the pretest and test periods, to minimize evaporation and to reduce the possibility of contamination. Any overlying water lost by evaporation should be replaced midway through the test (or more frequently, if desired or necessary) by the gentle addition of temperature-adjusted test water poured down the side of the test chamber. A 275-mL mark inscribed on the side of the test chamber can be used to judge this.

Test organisms used to begin the test are derived from laboratory cultures (i.e., egg masses obtained from cultured adults in preparation for the test; see Section 2.3.10). To start a test, third instar larvae of *C. tentans* or first instar larvae of *C. riparius*

must be used.<sup>24</sup> If *C. tentans* are used as test organisms, a minimum of 20 larvae selected randomly from organisms taken from the culture on Day 0 to begin a test must be measured (using a dissecting microscope with an ocular micrometer) for head capsule widths to confirm their instar. It is also desirable to measure their dry weight or length at this time (USEPA, 1994a). If *C. riparius* are used as test organisms, they must be first instars that are  $\leq 48$  h post-hatch. Head capsule measurements are not required on Day 0 for *C. riparius*, since organisms  $\leq 48$  h post-hatch are, without doubt, first instars. Table 3 provides guidance on the ranges of age and head capsule widths for the various larval stages of *C. tentans* or *C. riparius*.

In many instances, the culture water and the water used as overlying water in the test will be the same, although this is not necessarily so. The objectives of a particular test might require the use of another water source (e.g., that from a particular site under investigation) as test water. If water other than culture water is used as test water, acclimation of test organisms to this water is not required (USEPA, 1994a) although it might be advisable to do so in order to minimize any stress on the animals caused by different water quality characteristics. If test organisms are to be acclimated, a useful

procedure is to hold them for 2 h in a 50:50 mixture of culture water:test water, then for 2 h in a 25:75 mixture of culture water:test water, followed by a final 2 h in 100% test water before their introduction to test chambers (Ingersoll and Nelson, 1990). This should be done on the day before the test starts.

On Day 0, ten animals should be assigned randomly to each test chamber. The organisms should be handled as little and as carefully as possible (see Section 2.3.9) during their transfer from the culture chamber(s) to the test chambers. Midge larvae must be placed below the air/water interface in the overlying water. Test organisms may be pipetted directly from a culture chamber into the overlying water (Ankley *et al.*, 1993). Alternatively, 10 larvae may be counted into a transfer chamber (e.g., 30-mL plastic cup) filled with test water at the test temperature, and then recounted before their transfer below the surface of the overlying water (Ingersoll and Nelson, 1990; USEPA, 1994a). The latter procedure is particularly useful, since it permits the organisms to be counted twice before they are introduced to the test chamber. Any larvae that remain stuck to the transfer chamber may be washed carefully into the overlying water in the test chamber, using a Pasteur pipette and a minimal quantity of test water adjusted to the test temperature. Following the addition of test organisms, the volume of water overlying the sediment should be increased as necessary until the 275-mL mark inscribed on the chamber's side is reached.

<sup>24</sup> *C. tentans* must be third instar or younger at the start of a test, with at least 50% of the organisms at third instar, a practice in keeping with that of USEPA (1994a) and ASTM (1955a). First instar larvae must be used for starting 10-day sediment toxicity tests with *C. riparius*, because this species develops more rapidly than *C. tentans*. At 23°C, adult *C. riparius* can emerge as early as 15 to 16 days, depending on sex (Day *et al.*, 1994), too rapidly for a 10-day test unless it is started with first instars.



**Table 3** Age and Head Capsule Width of *Chironomus tentans*<sup>25</sup> and *Chironomus riparius*<sup>26</sup> Instars Reared at 23°C

| Species            | Instar | Days After Hatching  | Head Capsule Width (mm) |              |
|--------------------|--------|----------------------|-------------------------|--------------|
|                    |        |                      | Mean                    | Range        |
| <i>C. tentans</i>  | first  | 1.0 to 4.4           | 0.10                    | 0.09 to 0.13 |
| <i>C. tentans</i>  | second | 4.4 to 8.5           | 0.20                    | 0.18 to 0.23 |
| <i>C. tentans</i>  | third  | 8.5 to 12.5          | 0.38                    | 0.33 to 0.45 |
| <i>C. tentans</i>  | fourth | >12.5                | 0.67                    | 0.63 to 0.71 |
| <i>C. riparius</i> | first  | 0 to 3 <sup>27</sup> | 0.10                    | 0.07 to 0.12 |
| <i>C. riparius</i> | second | 4 to 6               | 0.19                    | 0.13 to 0.24 |
| <i>C. riparius</i> | third  | 7 to 14              | 0.33                    | 0.26 to 0.40 |
| <i>C. riparius</i> | fourth | >8                   | 0.55                    | 0.43 to 0.60 |

<sup>25</sup> As determined by Norberg-King (1994) and cited in USEPA (1994a).

<sup>26</sup> RIZA (1993) and Day *et al.* (1994), plus unpublished data of Dr. K.E. Day and co-workers (Aquatic Ecosystem Conservation Branch, National Water Research Institute, Environment Canada, Burlington, ON).

<sup>27</sup> Most larvae will have become second instars by four days post-hatch.



## 4.2 Test Conditions

- This is a whole sediment toxicity test, during which the overlying water is:
  - (1) not renewed except for the periodic (e.g., on Day 7) addition of required volumes of test water to replace that lost from evaporation (i.e., static test option); or
  - (2) is renewed throughout the test at a rate of two volume additions per day (i.e., daily-renewal test option).
- Test duration is 10 days.
- The test must be conducted at a daily mean temperature (overlying water) of  $23 \pm 1^\circ\text{C}$ . Additionally, the instantaneous temperature must be  $23 \pm 3^\circ\text{C}$  (USEPA, 1994a; ASTM, 1995a).<sup>28</sup>
- The recommended test container is a 300-mL high-form glass beaker or glass jar, with an inner diameter of ~7 cm.
- Control and test sediments must be present as a uniform, 100-mL layer, with a 175-mL volume of overlying water.
- For the daily-renewal test option, test chambers are normally uncovered, although covers may be used to minimize loss of volatiles from the sediment or to reduce the risk of contamination. Overlying water is normally not aerated unless its concentration of dissolved oxygen drops below 40% saturation at any time during the test (see Section 4.3). Using this test option, organisms in each test chamber must be fed either four times during the test (nonconsecutive days) or daily (see Section 4.4).
- For the static test option, test chambers should be covered. The overlying water in each chamber should be aerated continuously at a minimal rate (see Section 4.3). Organisms in each test chamber must be fed either four times during the test (nonconsecutive days) or daily (see Section 4.4).
- Test chambers are to be illuminated with a daily photoperiod of 16-h light and 8-h dark, using overhead, full-spectrum lights (fluorescent or equivalent). Light intensity adjacent to the surface of the overlying water should be 500 to 1000 lux.
- For a valid test, the mean survival rate for midge larvae in control sediment must be  $\geq 70\%$  at the end of the test. Additionally, the minimum

<sup>28</sup> Certain provincial (Bedard *et al.*, 1992) or other procedural documents (e.g., RIZA, 1992 and BBA/IVA, 1994) specify the use of a somewhat lower or more variable test temperature. Such an approach could be applied while using the guidance, procedures and conditions specified herein, provided that the test report clearly identifies such a deviation (see Section 7). The investigator should be aware, however, that a decreased test temperature might make it somewhat more difficult to achieve the minimum mean dry weight for controls at test end, which is specified here for each species of midge larvae as one of the requirements for a valid test.

dry weight for the replicate control groups at test end (Day 10) must average  $\geq 0.6$  mg per individual organism if *C. tentans* is used (USEPA, 1994a; ASTM, 1995a), or  $\geq 0.5$  mg if *C. riparius*<sup>29</sup> is used.

### 4.3 Dissolved Oxygen and Aeration

Midge larvae can survive hypoxic conditions, although their survival and/or growth can be adversely affected if dissolved oxygen concentrations are below about 25 to 35% for an extended period (USEPA, 1991a) (Section 1.4). Using the daily-renewal test option, aeration of the overlying water is normally not required. The dissolved oxygen concentration in the overlying water will not be below 40% saturation due to its daily renewal, unless the sediment sample used in the test has an unusually high oxygen demand. If at any time during a daily-renewal test the dissolved oxygen is below 40% saturation or above 100% saturation in one or more test chambers, the overlying water in all test chambers including the controls should be aerated to maintain its dissolved oxygen concentration between 40% and 100% saturation. If the overlying water is to be aerated, oil-free compressed air should be dispensed to each test chamber through airline tubing and a disposable

plastic or glass tube (e.g., capillary tubing or a pipette having an Eppendorf tip) with a small aperture (e.g., 0.5 mm ID). Stainless steel gang valves are useful for regulating air flow. The tip of the air delivery tube should be suspended approximately 3 cm above the surface of the sediment layer. Air flow to each test chamber must be gentle (e.g., 2 to 3 bubbles/s), and must not disturb the sediment surface (Zumwalt *et al.*, 1994). Any aeration during testing must be reported (Section 7).

Using the static test option, the overlying water in each test chamber should be aerated continuously on the night preceding the start of the test (see Section 4.1), as well as during the test. Compressed air, previously filtered so as to be free of oil, should be bubbled through a disposable glass pipette. The tip of the pipette should be suspended approximately 3 cm above the surface of the sediment layer. Air flow to each test chamber must be gentle and regulated to provide approximately 2 to 3 bubbles/s. The air flow to each test chamber should be checked routinely (e.g., daily) throughout the test, and adjustments made if necessary to maintain a gentle rate of aeration.

### 4.4 Food and Feeding

Using either test option, organisms in each test chamber must be fed either once daily throughout the test, or four times only (on nonconsecutive days) during the test. Since dry weight of animals is a primary endpoint for the test, an identical food ration must be added to each test chamber on each feeding occasion. The ration provided must be adequate to enable acceptable survival and growth of *C. tentans* or *C. riparius* during

<sup>29</sup> Based on a review of dry weight attained by control groups in 10-day tests with *C. riparius*, using under static or static-renewal conditions defined in this report, Milani *et al.* (1996) concluded that a criterion for test validity of  $\geq 0.5$  mg per individual control organism would normally be attainable yet discriminatory for this species, and recommended this for inclusion as a test criterion using either option.

the test period (see Section 4.2), but must not be excessive.<sup>30</sup>

If daily feeding is chosen, 6.0 mg (dry weight) of ground tropical fish food flakes (e.g., Tetrafin™ or Nutrafin™), must be added as a 1.5-mL inoculum of a 4.0 mg/mL suspension of dry food in water to each test chamber on Day 0, as well as once per day until the day the test ends. If the option of feeding four times only during the test is chosen, 15.0 mg (dry weight) of ground tropical fish food flakes must be added as a 3.75-mL inoculum of a 4.0 mg/mL suspension of dry food in water to each test chamber on Day 0, as well as once on each of three additional, nonconsecutive days during the test<sup>31</sup> until test completion. Either ration results in the same overall rate of feeding; i.e., 60.0 mg dry food added to each test chamber during the 10-day test period. Daily feeding is preferable to "even out" the available food supply, although feeding four

times only (nonconsecutive days) during the 10-day test might be a preferred choice to minimize weekend labour requirements (Milani *et al.*, 1996).

A single-ration diet of ground commercial fishfood flakes has been demonstrated to enable good survival and growth of *C. tentans* or *C. riparius* during 10-day tests (NWRI, 1992; OME, 1992; Ankley *et al.*, 1993; Call *et al.*, 1993a; USEPA, 1994a; Milani *et al.*, 1996). This diet provides a reasonably consistent nutritive value for midge larvae, and is simple to prepare. A daily ration of 6.0 mg (dry solids) of Tetrafin has been recommended by USEPA (1994a) for 10-day *survival-and-growth* tests with *C. tentans*, and this or the same ration of Nutrafin is also adequate for 10-day tests with *C. riparius* (Milani *et al.*, 1996).

A stock suspension of food for use during the test can be prepared by grinding (mortar and pestle) a flaked commercial fish food (e.g., Tetrafin or Nutrafin), sieving (e.g., using a 500 µm or #110 Nitex™ screen), and mixing the fines with deionized water for 1 to 3 min (NWRI, 1991; USEPA, 1994a). The blended slurry can then be placed in 100- to 500-mL screw-top plastic bottles for storage. Each batch of food should be refrigerated, and can be used for up to two weeks (USEPA, 1994a). The prepared food suspension should be thoroughly mixed before aliquots are taken.

Detailed records of the food type and ration added to each test chamber should be made on each feeding occasion. Observations of the appearance of the sediment surface in each test chamber (i.e., any evidence of a fungal or bacterial growth) should also be recorded at this time. If, using the daily-

<sup>30</sup> Feeding during the test is essential to enable adequate (≥ 70%) survival and acceptable growth of test organisms (Ankley *et al.*, 1993; 1994; Milani *et al.*, 1996). The addition of excess or different types of food is to be avoided since it might alter the bioavailability of contaminants in the sediment and/or promote the growth of fungi or bacteria on the sediment surface (USEPA, 1994a).

<sup>31</sup> For instance, if sediment and water were added to test chambers on a Monday and animals introduced the next day, they could be fed on Tuesday and Friday of that work week and on Monday and Wednesday of the next work week, with the test ending on a Friday. A side-by-side comparison of *C. riparius* fed an equivalent overall ration (i.e., 60.0 mg food/chamber, over 10 days), offered as either daily feedings of 6.0 mg dry weight or four times only feedings of 15.0 mg dry weight each, demonstrated that these differing patterns of feeding did not influence survival or growth during the 10-day test regardless of whether the static or the static-renewal options were used (Milani *et al.*, 1996).

renewal test option, the dissolved oxygen concentration in one or more chambers drops below 40% saturation, feeding in all treatments should be temporarily suspended for the amount of time necessary for its recovery (USEPA, 1994a).

#### 4.5 *Observations and Measurements During the Test*

If the daily-renewal test option is chosen and an automated water-renewal system is used (see Sections 3.1 and 4.1), its operation should be monitored daily. Any observations of water flow problems, or overflows in test chambers due to clogged drain screens, should result in immediate cleaning or other required maintenance.

Depending on the study objectives or the clarity of overlying water in test chambers, it might be worthwhile to check each test chamber daily (or less frequently), to observe and record the number of midge larvae seen on the surface of the sediment. The investigator should be aware that such observations, which are intended to indicate avoidance responses of test organisms, are sometimes difficult or impossible to undertake due to turbidity of the overlying water.

The temperature of the overlying water must be measured at the beginning of the test, and thereafter at least three times per week on nonconsecutive days (e.g., Mondays, Wednesdays, Fridays) until test completion. These measurements must be made in at least one test chamber representing each treatment; and more frequent (i.e., daily) measurements are recommended. Additionally, it is recommended that the

temperature of any water bath used, and/or of the air in a temperature-controlled room or chamber used for the test, be recorded continuously.

For at least one test chamber representing each treatment, the concentration of dissolved oxygen in the overlying water must be measured at the beginning of the test, and thereafter at least three times per week on nonconsecutive days (e.g., Mondays, Wednesdays, Fridays) until test completion. More frequent (e.g., daily; ASTM, 1995a) measurements might be advisable and would be warranted for sediments with a high oxygen demand. A probe and calibrated dissolved oxygen (DO) meter is recommended for these measurements. The probe must be inspected carefully after each reading to ensure that organisms have not adhered to it, and must be rinsed in deionized or distilled water between samples to minimize cross-contamination. If beakers or jars are aerated during the test (Section 4.3), the position of the tip of the pipette in each test chamber and the rate of aeration should be checked frequently and routinely, and adjustments made as necessary.

Conductivity, pH, and ammonia concentrations in the overlying water must be measured at the beginning and end of the test for at least one test chamber representing each treatment. Additionally, hardness and/or alkalinity concentrations in the overlying water should be measured at the beginning and end of the test in at least one test chamber representing each treatment (USEPA, 1994a). For each measurement of ammonia (see APHA *et al.*, 1995 for guidance), the concentration of unionized ammonia should be calculated based on the



concurrent measurements of pH and temperature for the overlying water (Trussell, 1972; USEPA, 1985c).

Conductivity and pH may be measured using probes and calibrated meters. Ammonia may be measured using an ion-specific electrode or by extracting an aliquot of the overlying water for this analysis. As with DO measurements, any probe inserted in a test chamber must be inspected carefully immediately after each reading, and rinsed in deionized water between samples. For measurements of hardness, alkalinity, and ammonia requiring sample aliquots, samples of overlying water should be taken just before the addition of test organisms and upon completion of the test. For these samples, it might be necessary to pool water samples from individual replicates. No more than 10% of the volume of the overlying water in a test chamber (i.e.,  $\leq 17.5$  mL) should be removed for this purpose. A pipette should be used carefully to remove water from a depth of about 1 to 2 cm above the sediment surface.<sup>32</sup> The pipette should be checked to make certain that no midge larvae are removed during the collection of these water samples.

The water quality measurements determined at the beginning and end of a test for each treatment are useful as they provide an indication of the influence of the sediment on overlying water quality during the test. If, for any treatment, a marked change (e.g.,  $>50\%$ ; USEPA, 1994a) in one or more of these water-quality variables is found between the initial and final measurements, a check on the conditions and procedures used

in the test (e.g., rate of renewal of overlying water, if a daily-renewal test) is recommended, together with a careful consideration of the physicochemical characteristics of the sediment used in the test.

#### 4.6 *Ending a Test*

The test is terminated after ten days. Just before sieving the contents of a test chamber, all live and apparently dead midges on the surface of the sediment should be pipetted from the test chamber. Individuals which are completely inactive but not obviously dead (e.g., not decomposing) should be held in test water within a petri dish or other suitable container, and examined closely at this time using a low-power microscope or hand-held magnifying glass. These individuals should be prodded gently with a sharp point to confirm that they show no sign of life, and are then to be counted as dead.

Numbers of dead and surviving organisms recovered by pipetting should be recorded. Dead animals should be discarded. All live animals should be placed in a numbered weighing boat or similarly small holding receptacle. This receptacle should contain a volume of test water sufficient for rinsing the larvae and holding them briefly until all of the survivors sieved from the sediment in the test chamber are added to and rinsed in it.

A consistent amount of time should be taken to sieve the contents of each test chamber and examine this closely for recovery of live or dead organisms. To ensure that the procedure used to recover organisms is adequate, it is recommended that the laboratory personnel responsible for sieving

<sup>32</sup> It might be necessary to pool water samples from individual replicates.



the contents of test chambers demonstrate that they are able to retrieve an average of at least 90% of *Chironomus* sp. from sediment.<sup>33</sup>

The following technique, taken from USEPA (1994a), is recommended for sieving the contents of each test chamber. Other techniques or mesh sizes (e.g., 250  $\mu\text{m}$ , for *C. riparius* and perhaps also *C. tentans*) may also be used provided that they have been demonstrated in preliminary trials to allow the retrieval of test organisms.

- (1) Pour approximately 50% of the overlying water through a #50 (300  $\mu\text{m}$ ) U.S. Standard mesh sieve.
- (2) Swirl the remaining water to suspend the upper ~1 cm of sediment. Pour the suspended slurry through the #50 mesh sieve. Using test water, wash the contents recovered on the sieve into a white tray or pan for inspection.
- (3) Using test water and a wash bottle, rinse the coarser sediment remaining in the test chamber through a #40 (425  $\mu\text{m}$ ) mesh sieve and wash the contents recovered on this sieve into a second tray or pan for inspection.

Before drying organisms for weight determination, it is recommended that the head capsule width of each surviving larva recovered from each test beaker be measured

using a dissecting microscope and ocular micrometer. These measurements enable an assessment of instar development during the 10-day exposure.<sup>34</sup>

All live animals recovered from the overlying water or sediment in a single test chamber are counted and placed together in a numbered weighing boat or similar small holding receptacle, and rinsed in test water to remove any sediment adhering to the animal. The rinse should be brief, and no more than 10 min after introduction of the first animal. After rinsing, the group of surviving midge larvae should be transferred to a clean, aluminum weighing boat that has been previously numbered, weighed, and held in a desiccator.<sup>35</sup>

Separate weighing boats, each containing the group of surviving midge larvae recovered from a given test chamber (replicate), are placed in an oven, and dried for 24 h at 60°C (NWRI, 1992). Upon removal from the oven, the boats are moved immediately to a desiccator. Following cooling, each boat should be removed from the desiccator, and

<sup>33</sup> USEPA (1994a) recommends a check on recovery capability used by Tomasovic *et al.* (1995), whereby test organisms are added to control sediment and their recovery determined after 1 h using the same technique as that employed for sieving the contents of test chambers at the end of the test.

<sup>34</sup> Retardation of instar development has been reported under conditions of toxicity (Powlesland and George, 1986; Pascoe *et al.*, 1990). Measurements of head capsule width will enable an evaluation of a sublethal effect of the sediment exposure on larval development, and will be useful in assessing if lower weights at Day 10 are associated with slower instar development (Day *et al.*, 1994).

<sup>35</sup> It might be advisable to oven-dry the weigh boats for at least 48 h to achieve a constant weight, since wax deposits associated with the weigh boats could otherwise provide weighing errors (G.T. Ankley, USEPA Environ. Res. Lab., Duluth, MN, personal communication, 1994).

weighed immediately<sup>36</sup> to the nearest 10 µg on a balance that measures accurately to this limit. Mean dry weight per organism which survived the test is calculated for each group<sup>37</sup> (see Section 4.7).

<sup>36</sup> The dried organisms can take up water vapour readily, so weighing should be rapid and the time standardized among boats. At the same time, care must be taken because rapid movement and static charge could cause dried specimens to be lost from the weighing boat.

<sup>37</sup> The body length of individual organisms surviving at the end of the test has been used as an alternative measurement of growth in sediment toxicity tests with midge larvae (see Appendix D.10). Herein, dry weight is the recommended indicator of growth. Measurement of fresh (wet) weight of individual organisms might also be useful, especially for tests including organoleptic analyses for contaminants (Bedard *et al.*, 1992; Bedard, 1996); tests including wet-weight measurements require additional control groups to determine if the criterion for test validity based on mean dry weights of individual controls is met (Sections 4.2 and 4.6). Measurement of body length offers some additional advantage over dry-weight measurements, in that specimens can be preserved for subsequent analyses (USEPA, 1994a), and data derived from individuals (rather than for the group, as is the case for measurements of weight) can be used for nested ANOVA (Kemble *et al.*, 1994). Length determinations can also be automated using a computer-interfaced digitizing system coupled with a microscope (USEPA, 1994a). Measurements of length can be substituted in this test for dry-weight measurements provided that future studies with midge larvae demonstrate conclusively that length is as sensitive an indicator of growth, or more sensitive. Results by Becker *et al.* (1995) provide supporting evidence in this regard. In Environment Canada's *survival-and-growth* test using fathead minnows (EC, 1992d), attained size is assessed by mean dry weight alone; length is not used as a criterion of effect due to evidence that increased body depth and weight of healthy individuals is not adequately reflected in gains in body length during the test. A similar phenomenon could occur during the present 10-day *survival-and-growth* test using midge larvae.

During the series of dry-weight determinations for the groups of midge larvae from a test, the first boat weighed should be replaced in the desiccator and weighed again at the end of all weighings, as a check on gain of water by the boats in the dessicator to be weighed subsequently. The change should not be >5%; if it is, redrying of all boats for ≥2 h and reweighing might be carried out. A few weighing boats should be tared, dried, and weighed without organisms, and results should conform to the laboratory's quality control standards.

#### 4.7 Test Endpoints and Calculations

The biological endpoints for this 10-day sediment toxicity test are survival and dry weight. Reduced survival and/or lesser weight at test end is assessed by comparison with replicate reference and/or control groups (see Sections 5.6 and 6.5). The most sensitive of the two effects is taken as the definitive indication of toxicity.

At the end of the 10-day exposure, the number of midge larvae alive and number dead are recorded for each replicate including the control groups. Missing individuals are assumed to have died and disintegrated during the test, and are included in the tally of dead individuals for a replicate. The total weight of the group of survivors in a replicate is measured.

The following two endpoints must be calculated for each treatment:

- the mean (± SD) percentage of organisms that survived the exposure.

- the mean ( $\pm$  SD) dry weight per surviving animal, calculated from the total weight of the group of survivors.

Calculation of the mean ( $\pm$  SD) head capsule width for the group of survivors in each treatment is also recommended as a useful endpoint (Section 4.6).

The test is invalid if the average percent survival of organisms in the control sediment is  $<70\%$ . The test is also invalid if the mean dry weight of surviving control organisms is  $<0.6$  mg per individual *C. tentans* or  $<0.5$  mg per individual *C. riparius* (Section 4.2).

Various statistical procedures can be used to assess the results of the test. The options, rationale for choice, and methods of calculation are discussed in depth in reports by the USEPA (1989; 1994a; b) and Environment Canada (EC, 1997d). The choice of statistical treatment depends on the study design and, in particular, whether tests used replicates of sediment or multiple concentrations of test materials or substances. Sections 5 and 6 provide guidance on statistical endpoints and calculations.

#### 4.8 Tests with Reference Toxicant(s)

The routine use of a reference toxicant or toxicants is necessary to assess, under standardized test conditions, the relative sensitivity of the culture(s) of *C. tentans* or *C. riparius* and the precision and reliability of data produced by the laboratory. *Water only* tests with one or more reference toxicants are most commonly used in conjunction with *survival-and-growth* tests

which measure sediment toxicity to midge larvae (see Appendix E). Procedures for spiking sediment with reference chemical(s) are being developed (Burton, 1991; Suedel *et al.*, 1993a; b) and should see wider use in the future. A static, 96-h *water-only* reference toxicity test is recommended here for routine use with sediment tests using midge larvae, a practice followed by USEPA (1994a). This reference toxicity test may be supplemented or replaced with one or more *spiked-sediment* tests with reference toxicant(s) after suitable procedures are standardized. Environment Canada's guidance document on using control sediment spiked with a reference toxicant should be consulted (EC, 1995).

Table 4 provides a checklist of conditions and procedures recommended for conducting static, 96-h *water only* reference toxicity tests using *C. tentans* or *C. riparius*. The recommended test procedure, which is largely consistent with USEPA (1994a), uses second instar midge larvae of either species to start the test. There are ten individuals per test chamber, at least five test concentrations plus a control (i.e., using control/dilution water only), and one or more replicates per treatment. Recommended test chambers are 300-mL high form glass beakers or glass jars with an inner diameter of  $\sim 7$  cm, and the recommended test volume is 200 mL solution/chamber. Solutions in test chambers are not aerated during the test and are normally covered to minimize contamination and losses due to evaporation. A 1.25-mL volume of a suspension of finely ground commercial fish food flakes (e.g., Tetrafin<sup>TM</sup> or Nutrafin<sup>TM</sup>), comprised of 4.0 mg of dry solids/mL (see Section 4.4), should be added to each test chamber on Days 0 and 2 of the test (USEPA, 1994a).

**Table 4** Checklist of Recommended Conditions and Procedures for Conducting Water Only Reference Toxicity Tests Using *Chironomus* sp.

|                               |                                                                                                                                                                                |
|-------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Test type                     | - static 96-h water only toxicity test                                                                                                                                         |
| Reference toxicant            | - copper sulphate (CuSO <sub>4</sub> ), cadmium chloride (CdCl <sub>2</sub> ), potassium chloride (KCl), or sodium chloride (NaCl)                                             |
| Frequency of test             | - once per month, or in conjunction with definitive test(s) with sediment samples                                                                                              |
| Test solutions                | - control and at least 5 test concentrations                                                                                                                                   |
| Solution replacement          | - none                                                                                                                                                                         |
| Control/dilution water        | - culture water or other clean ground or surface water; reconstituted fresh water if a high degree of standardization is required; DO, 90 to 100% saturation when used in test |
| Test organisms                | - <i>C. tentans</i> or <i>C. riparius</i> ; second instar at start of test; 10/test chamber                                                                                    |
| Substrate for organisms       | - monolayer of silica sand                                                                                                                                                     |
| Test chamber                  | - 300-mL high form glass beaker or glass jar, ~7 cm ID; normally covered                                                                                                       |
| Volume of test solution       | - 200 mL                                                                                                                                                                       |
| Number of replicates          | - one or more per concentration                                                                                                                                                |
| Temperature                   | - daily average, 23 ± 1 °C; instantaneous, 23 ± 3 °C                                                                                                                           |
| Lighting                      | - overhead full-spectrum (fluorescent or equivalent); 500 to 1000 lux; 16-h light:8-h dark                                                                                     |
| Aeration                      | - none                                                                                                                                                                         |
| Feeding                       | - ground commercial fish food flakes (e.g., Tetrafin™ or Nutrafin™); 1.25 mL suspension <sup>38</sup> fed to each chamber on Days 0 and 2                                      |
| Observations                  | - daily, each chamber, for number of dead or moribund organisms                                                                                                                |
| Measurements of water quality | - daily, each treatment, for DO and temperature; start and end of test, each treatment, for pH, alkalinity, hardness, conductivity                                             |
| Endpoints                     | - mean percent survival, each treatment; 96-h LC50                                                                                                                             |
| Test validity                 | - results for reference toxicity test considered invalid if mean 96-h survival in control water <90%                                                                           |

<sup>38</sup> Each 1.0 mL of suspension contains 4.0 mg of dry solids.

Temperature and lighting conditions are the same as those for definitive sediment toxicity tests (see Section 4.2 and Tables 2 and 4). Daily observations are made for numbers of dead or moribund midge larvae in each chamber. Temperature and dissolved oxygen are measured daily for each treatment; and pH, alkalinity, hardness, and conductivity are measured for each treatment at the start and end of the test (Section 4.5). The endpoints are mean percent survival for each treatment, and the 96-h LC50. Results for a reference toxicity test must be declared invalid if the mean survival of midges in control water is <90% at the end of the test (Table 4).

Appropriate criteria for selecting suitable reference toxicant(s) might include the following (EC, 1990b; 1995):

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- can be interspersed evenly throughout clean substrate;
- good dose/response curve for test organism;
- stable in aqueous solution;
- minimal hazard posed to user;
- concentration easily measured with precision;
- known influence of water quality (e.g., pH, hardness) on toxicity of chemical to test organism; and

- known influence of physicochemical characteristics of sediment (e.g., particle size, organic carbon content) on toxicity of chemical to test organism.

Reagent-grade copper sulphate, cadmium chloride, potassium chloride, or sodium chloride are recommended for use as reference toxicants with *C. tentans* or *C. riparius* (USEPA, 1994a). Reference toxicity tests using *Chironomus* sp. and one or more of these chemicals must be performed monthly with the laboratory's established cultures, if sediment toxicity tests with midge larvae are being performed frequently (e.g., weekly to once monthly) and routinely by the laboratory. The performance of any cultures recently established in the laboratory using new breeding stock should also be evaluated using reference toxicant(s) before these cultures are used to provide test organisms (see Sections 2.3.1 and 2.3.11).

Pertinent reports by Environment Canada provide guidance on the selection, performance, and use of *water only* (EC, 1990b) or *spiked-sediment* (EC, 1995) reference toxicity tests. Laboratory personnel unfamiliar with such tests are advised to consult these reports before preparing for or conducting them.

It is the laboratory's responsibility to demonstrate its ability to obtain consistent, precise results with reference toxicant(s) before conducting definitive sediment assays with *C. tentans* or *C. riparius*. To meet this responsibility, the laboratory personnel should initially determine intralaboratory



precision, expressed as coefficient of variation (CV), by performing five or more tests with the reference toxicant(s) using different groups of *C. tentans* or *C. riparius* from separate cultures reared in their laboratory (USEPA, 1994a). For these preliminary tests, the same reference toxicant(s), concentrations, type/source of test water, and test procedure (see Table 4) should be used. Performance of the routine (monthly) tests with reference toxicant(s) should continue to follow this same procedure. A series of test concentrations should be chosen<sup>39</sup> on the basis of preliminary tests, to provide partial mortalities in two or more concentrations and enable calculation of a 96-h LC50 with acceptably narrow confidence limits (see Section 6.5).

Once sufficient data are available (EC, 1990; 1995), LC50s for a particular reference toxicant must be plotted successively on a warning chart and examined to determine whether the results are within  $\pm 2$  SD of values obtained in previous tests using the same reference toxicant and test procedure. A separate warning chart must be prepared and updated for each reference toxicant used. The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new LC50 for the reference toxicant should be compared with established limits of the chart; the LC50 is acceptable if it falls within the warning limits.

The logarithm of concentration (including LC50) should be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each LC50 was estimated based on logarithms of concentrations. The warning chart may be constructed by plotting the logarithmic values of the mean and  $\pm 2$  SD on arithmetic paper, or by converting them to arithmetic values and plotting those on the logarithmic scale of semi-log paper. If it were demonstrated that the LC50s failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable.

The mean of the available values of log(LC50), together with the upper and lower warning limits ( $\pm 2$  SD), should be recalculated with each successive LC50 for the reference toxicant until the statistics stabilize (EC, 1990; 1995; 1997d). If a particular LC50 fell outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5% of the time due to chance alone, an outlying LC50 would not necessarily indicate abnormal sensitivity of the culture or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A thorough check of all culturing and test conditions and procedures should be carried out. Depending on the findings, it might be necessary to repeat the reference toxicity test, to obtain new breeding stock, and/or to establish new known age cultures, before undertaking further sediment toxicity tests.

<sup>39</sup> See Appendix H for guidance in selecting an appropriate series of test concentrations. Each successive concentration chosen should be at least 50% of the previous concentration.

Results that remained within the warning limits might not necessarily indicate that a laboratory was generating consistent results.

Extremely variable data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variation

in test results. A coefficient of variation of no more than 30%, and preferably 20% or less, is suggested as a reasonable limit by Environment Canada (1990).

## Section 5

---

# Specific Procedures for Testing Field-collected Sediment or Similar Particulate Material

This section gives particular instructions for preparing and testing samples of field-collected sediment or similar particulate material. These instructions are in addition to the procedures listed in Section 4. Toxicity tests with these samples should be conducted using either the daily-renewal test option or the static test option (see Section 4), depending on the test objectives or constraints and on any related regulatory guidelines or requirements.

Detailed guidance for the collection, handling, transport, storage, and analyses of field-collected sediment is given in ASTM (1991b, 1995b) and Environment Canada (1994) reports specific to these subjects. Environment Canada (1994) should be consulted and followed (in addition to the guidance provided here), when collecting samples of field-collected sediment and preparing them for toxicity tests with *C. tentans* or *C. riparius*.

## 5.1 Sample Collection

Environment Canada (1994) provides a useful summary of field-sampling design and appropriate techniques for sample collection. Field surveys of sediment toxicity using biological tests with *Chironomus* sp. and/or other suitable, sediment associated test organisms are frequently part of more comprehensive surveys. Such surveys could include a battery of toxicity tests to evaluate the acute or chronic toxicity of sediment, pore water, or elutriate, together with tests

for bioaccumulation of contaminants, chemical analyses, biological surveys of epifaunal and/or infaunal organisms, and perhaps the compilation of geological and hydrographic data. Statistical correlation can be improved and costs reduced if the samples are taken concurrently for these tests, analyses, and data acquisitions.

Samples of sediment collected for assessment of an adverse effect on survival and growth of *C. tentans* or *C. riparius* might be routinely taken (e.g., quarterly, semi-annually, or annually) from a number of sites for monitoring and compliance purposes, or might be collected on one or more occasions during field surveys of sites for spatial (i.e., horizontal or vertical) or temporal definition of sediment quality. One or more sites should be sampled for reference (presumably clean) sediment during each field collection.<sup>40</sup>

The number of stations to be sampled at a study site and the number of replicate samples per station will be specific to each study. This will involve, in most cases, a compromise between logistical and practical

---

<sup>40</sup> A reference sediment is that collected near the site(s) of concern. Ideally, it possesses geochemical characteristics similar to those of the test sediment but without anthropogenic contaminants. It is not unusual for nearby reference sites to have some degree of contamination due to anthropogenic chemicals. In some instances, reference sediment might be toxic due to naturally occurring physical, chemical, or biological properties (Burton, 1991).

constraints (e.g., time and cost) and statistical considerations. Environment Canada (1994) should be consulted for guidance with respect to the sampling design, including the recommended minimum number of field replicates.

For certain monitoring and regulatory purposes, multiple field replicates (i.e., separate samples from different grabs or cores taken at the same site) should be taken at each sampling station, including one or more reference stations (EC, 1992a; 1994; 1997a). Each of these field replicates must be tested for its toxicity to *C. tentans* or *C. riparius*, using five or more test chambers per replicate sample. The use of *power analysis* statistics (see Section 5.6) with endpoint data obtained in previous *survival-and-growth* tests at the same or similar sites will assist in determining if more than five laboratory replicates need to be tested with each field replicate. Also, some of the statistical tests have requirements for minimum numbers of replicates. For certain other purposes (e.g., preliminary or extensive surveys of the spatial distribution of toxicity), the survey design might include only one sample from each station, in which case the sample would normally be homogenized and split between five or more test chambers (i.e., laboratory replicates). The latter approach precludes any determination of mean toxicity at a given sampling location (station), but allows a statistical comparison of toxicity of each sample with the control, and also if desired, a comparison among the test samples (stations), using appropriate statistical tests.

Sites for collecting reference sediment should be sought where the geochemical

properties of the sediment are similar to sediment characteristics encountered at the test sites. Marked differences in sediment characteristics including grain size or organic content might influence test results (see Section 1.4). Such possible effects should be considered when designing a study involving this biological test method. Matching of organic carbon content might not be warranted in cases where pollution (e.g., from pulp mills or sewage) is responsible for the high organic content of test sediments. Preliminary surveys to assess the geochemical properties and toxicity of sediment within the region(s) of concern and at neighbouring sites are useful for selecting appropriate sites at which to collect reference sediment.

Samples of municipal or industrial sludge (e.g., sewage sludge, dewatered mine tailings, sludge from an industrial clarifier or settling pond) might be collected for assessment of their adverse effect on survival and growth of *C. tentans* or *C. riparius*, and for geochemical and contaminant analyses. Other particulate wastes (e.g., drilling mud residue) might also be taken for toxicity and chemical evaluation.

Procedures used for sample collection (i.e., core, grab, dredge, or composite) will depend on the study objectives and the nature of the sediment or other particulate material being collected. The types of sediment collection devices and their advantages and disadvantages have been summarized by Environment Canada (1994), and further details are provided elsewhere (de Groot and Zschuppe, 1981; Baudo *et al.*, 1990; Burton, 1992; Sly and Christie, 1992; ASTM, 1995b).

A benthic grab or core rather than a dredge should be used for sampling sediment, to minimize disruption of the sample. Sediment to be evaluated for toxicity and chemistry should be collected from one or more depths that represent the layer(s) of concern (e.g., a surficial 2-cm layer, or a deeper layer if there are concerns about historical deposition of contaminants).

Care must be taken to minimize loss of fines during sample collection. If the sample is obtained using a grab sampler, hand corers should be used to collect a sample from the surficial 2 cm, or desired layer, of the test sediment retrieved using this sampling device. This can be achieved if the grab can be opened from the top to expose the surface of the undisturbed sediment. The sample should be transferred to a clean glass or plastic sample container.

Before commencing a sampling program, the required volume of sediment per sample should be calculated (EC, 1994). This calculation should take into account the quantity of sediment required to prepare laboratory replicates for sediment toxicity tests, as well as that required for particle size characterization, percent organic matter, percent moisture, and specific chemical analyses. A volume of at least 5 to 7 L of sediment per sample is normally required (EC, 1994); although this will depend on the study objectives/design and the nature of the chemical analyses to be performed. To obtain the required sample volume, it is frequently necessary to combine subsamples retrieved using the sampling device. Guidance provided in Environment Canada (1994) for compositing subsamples in the field should be followed.

The same collection procedure should be used at all field sites sampled. Environment Canada (1994) should be consulted for further guidance on appropriate devices and procedures for sample collection.

## 5.2 *Sample Labelling, Transport, Storage, and Analyses*

Containers for transport and storage of samples of field-collected sediment or similar particulate material must be made of nontoxic material. The choice of container for transporting and storing samples depends on both sample volume and the potential end uses of the sample. Environment Canada (1994) should be consulted for guidance in selecting suitable containers. The containers must either be new or thoroughly cleaned, and rinsed with test water or other clean water (e.g., distilled or deionized water) before use.

Each sample container should be filled completely, to exclude air. Immediately after filling, each sample container must be sealed and labelled or coded. Labelling and accompanying records made at this time must include at least a code or description which identifies sample type (e.g., grab, core, composite), source, precise location (i.e., water body, latitude, longitude, depth), replicate number, and date of collection; and should include the name and signature of sampler(s). Persons collecting samples of sediment should also keep records which describe details on:

- the nature, appearance, volume of each sample;



- the sampling procedure and apparatus;
- any procedure used to composite or subsample grabs or cores in the field;
- the number of replicate samples taken at each sampling station;
- the sampling schedule;
- the types and numbers of containers used for transporting samples; any field measurements (e.g., temperature, pH, dissolved oxygen) of the overlying water or sediment at the collection site; and
- procedures and conditions for cooling and transporting the samples.

Upon collection, warm ( $>7^{\circ}\text{C}$ ) samples should be cooled to between 1 and  $7^{\circ}\text{C}$  with regular ice or frozen gel packs, and kept cool ( $4 \pm 3^{\circ}\text{C}$ ) in darkness throughout the period of transport. As necessary, gel packs, regular ice, or other means of refrigeration should be used to assure that the temperature of the sample(s) remains within 1 to  $7^{\circ}\text{C}$  during transit.

The date of receipt of the sample(s) at the laboratory must be recorded. Sample temperature upon receipt at the laboratory should also be measured and recorded. Samples to be stored for future use must be held in airtight containers and in darkness at  $4 \pm 2^{\circ}\text{C}$  (EC, 1994; 1997a). Any air "headspace" in the storage container should be purged with nitrogen gas, before capping tightly (EC, 1994). Samples must not freeze or partially freeze during transport or

storage, and must not be allowed to dry (EC, 1992a; 1994; 1997a; b). It is recommended that samples of sediment or similar particulate material be tested as soon as possible after collection. The sediment toxicity test should begin within two weeks of sampling, and preferably within one week; the test must start no later than six weeks after sample collection.<sup>41</sup>

Ideally, sediment characteristics that are unstable (e.g., pH, oxidation-reduction potential) or changed by conditions of transit and storage (e.g., temperature) should be measured in the field to help characterize the sample. In the laboratory, each sample of field-collected sediment should be thoroughly mixed (Section 5.3), and representative subsamples taken for physicochemical characterization. Each sample (including all samples of control and reference sediment) must be characterized by analyzing subsamples for at least the following (USEPA, 1994a): for whole

---

<sup>41</sup> The toxicity and geochemistry of contaminated sediments from Hamilton Harbour was reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al.*, 1990). A study by Othoudt *et al.* (1991) found that the toxicity of samples of freshwater sediment did not differ significantly when stored at  $4^{\circ}\text{C}$  for periods of 7 to 112 days. Burton (1991) and USEPA (1994a) report studies by various researchers showing in some instances, that the toxicity of sediment held at  $4^{\circ}\text{C}$  was unchanged after several months' storage, and in other cases that changes were noted within days to weeks. A recommendation for testing within two weeks conforms with the advice in other sediment toxicity tests by Environment Canada (1992a, 1997a; b). A maximum permissible storage time of six weeks has been recommended by Environment Canada (1994) for sediments intended for toxicity tests, in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

sediment — particle size distribution (percentage of coarse-grained sand, medium-grained sand, fine-grained sand, silt, and clay), percent water content, and total organic carbon content; for pore water — pH and ammonia (total and un-ionized concentrations; see Section 4.5). Other analyses could include (USEPA, 1994a; APHA *et al.*, 1995): total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and porewater analyses for various physicochemical characteristics. Unless indicated otherwise, identical chemical, physical, and toxicological analyses should be performed with subsamples representative of each replicate sample of field-collected sediment (including reference sediment) taken for a particular survey of sediment quality, together with one or more subsamples of control sediment.

### 5.3 Preparing Sample for Testing

Field-collected sediment or similar particulate waste material should normally not be prepared for testing by sieving with water, as this would remove contaminants present in the pore water or loosely sorbed to particulate material (EC, 1994). Large debris or large indigenous macro-organisms should normally be removed using forceps or a gloved hand.

The presence of indigenous macro-organisms in samples can reduce the growth of midge larvae in freshwater sediment toxicity tests, and can confound the interpretation of test results (Reynoldson *et al.*, 1994). If a field-collected sediment contains a large number

of indigenous macro-organisms which cannot be removed using forceps, the sample may be press-sieved (not washed) through one or more suitably sized mesh screens. For those sediments containing small macro-organisms which, due to sediment characteristics cannot be removed by press-sieving, the sample(s) may be rinsed through a fine mesh sieve (e.g., 0.25 to 0.5 mm; Day *et al.*, 1995b) using any liquid that has separated from the sample during its transport and/or storage. This liquid must be remixed within the sieved sample (Section 4.1).

Sieving could alter the concentration or bioavailability of contaminants in the sediment, or alter its nutrient content and/or particle size (EC, 1994; Day *et al.*, 1995b). Therefore, if sediments are sieved, it is recommended that the physicochemical properties of the sediment (e.g., porewater metals, particle size distribution) be documented before and after sieving. Comparative toxicity tests using sieved and unsieved sediment might, in some cases, also be necessary or appropriate to discern the effect of sieving on sample toxicity.

Unless research or special study objectives dictate otherwise, each sample of field-collected test material should be homogenized in the laboratory before use (EC, 1994; USEPA, 1994a).<sup>42</sup> Mixing can affect the concentration and bioavailability of contaminants in the sediment, and sample

---

<sup>42</sup> One of the reasons for routinely homogenizing samples is to mix, into the sediment, any pore water which rises to the surface during sample shipment and storage. Homogenization is also necessary to redistribute the sample constituents that have compacted and layered according to particle size during transport and storage.

homogenization might not be desirable for all purposes.

To achieve a homogeneous sample, either mix it in its transfer/storage container, or transfer it to a clean mixing container. The sample may be stirred, using a nontoxic device (e.g., stainless steel spoon or spatula), until its texture and colour are homogeneous (EC, 1992a). Alternatively, a mechanical method (EC, 1994; USEPA, 1994a) may be used to homogenize the sample. For each sample included in a test, mixing conditions including duration and temperature must be as similar as possible. If there is concern about the effectiveness of sample mixing, subsamples of the sediment should be taken after mixing, and analyzed separately to determine homogeneity.

Immediately following sample mixing, subsamples of test material required for the toxicity test and for physicochemical analyses must be removed and placed in labelled test chambers (Section 4.1), and in the labelled containers required for storage of samples for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using *Chironomus* sp. or other test organisms should also be transferred to labelled containers at this time. All subsamples to be stored should be held in sealed containers with no air space, and must be stored in darkness at  $4 \pm 2^\circ\text{C}$  (Section 5.2) until used or analyzed. Just before it is analyzed or used in the toxicity test, each subsample must be thoroughly remixed to ensure that it is homogeneous.

## 5.4 Test Water

For tests with field-collected sediment or similar particulate material, the water introduced to test chambers (i.e., overlying water) may be from the same source as that used for culturing midge larvae (see Sections 2.3.4 and 3.4). Alternatively, this water may be from a separate supply of natural or reconstituted fresh water. For certain applications, the experimental design might require or endorse the use of fresh water taken from the reference site nearby where test sediments were collected. Use of uncontaminated site water, or uncontaminated water adjusted to the hardness of site water, is frequently a good choice due to the modifying influence of waters with different hardness values on the toxicity of metals or organic contaminants in sediment. Section 2.3.4 provides pertinent guidance on the preparation and analysis of water to be used as overlying water in the test.

## 5.5 Test Observations and Measurements

A qualitative description of each field-collected test material should be made when the test is being set up. This might include observations of sample colour, texture, and homogeneity; and the presence of plants, animals, and tracks or burrows of animals (EC, 1992a; 1997a). Any changes in the appearance of the test material and in the overlying water, observed during the test or upon its termination, should be noted and reported.

Measurements of the quality of the overlying water (e.g., pH, temperature, hardness, alkalinity, ammonia, dissolved oxygen content) in test chambers should be made during or at the beginning and end of the test, as described in Section 4.5. Depending on the test objectives and experimental design, separate test chambers might also be set up at the beginning of the test (Section 4.1) to monitor whole sediment and/or porewater chemistry (USEPA, 1994a). These would be destructively sampled during and at the end of the test. Test organisms might or might not be added to these extra test chambers, depending on study objectives. Measurements of chemical concentrations in the sediment or pore water within these chambers may be made by siphoning most of the overlying water without disturbing the surface of the sediment, then removing aliquots of the sediment for the appropriate analyses (see Section 5.2). If pore water were to be analyzed, centrifugation without filtration would be the recommended sampling procedure (USEPA, 1994a; EC, 1994). Environment Canada (1994) should be consulted for guidance on the recommended procedure for extracting pore water, and its treatment and storage before analyses.

Depending on study objectives and the nature of the test sediments (e.g., rich in organics), measurements of porewater pH and ammonia concentrations might be made as the test progresses, using test chambers dedicated for this purpose (EC, 1994; USEPA, 1994a). Other sediment characteristics (e.g., concentrations of metals, hydrogen sulphide, total volatile solids, Eh) might be monitored in the same test chambers. If it were desired to monitor these variables, at least one chamber should

be set up for each treatment, and destructively sampled for this purpose.

## 5.6 *Test Endpoints and Calculations*

The common theme for interpreting tests with samples of sediment, is a comparison of the biological effects with the effects found in a reference sediment. The reference sample should be used for comparative purposes whenever possible or appropriate, because this provides a site-specific evaluation of toxicity (USEPA, 1994a). Sometimes the reference sediment might be unsuitable for comparison because of toxicity or atypical physicochemical characteristics. In such cases, it would be necessary to compare the test sediments with the control sediment. The control sediment(s) results will assist in distinguishing contaminant effects from noncontaminant effects caused by such things as particle size and organic carbon content. Regardless of whether the reference sediment or control sediment is used for the statistical comparisons, the results from control sediment must be used to judge the validity and acceptability of the test (Section 4.2).

Analysis of results will differ according to the purposes and particular designs of the test. This section covers the analytical procedures, starting with the simplest design and proceeding to the more complex designs. Standard statistical procedures are generally all that is needed for analyzing the results. Investigators should consult Environment Canada (1997d) as well as USEPA (1994a, Section 14) and USEPA/USACE (1994; Appendix D) for guidance on the appropriate statistical endpoints and their calculation. As always, advice of a statistician familiar with



toxicology should be sought for design and analysis of tests.

Analysis of variance (ANOVA) and multiple-comparison tests are commonly used for statistical interpretation of sediment tests. This is an hypothesis-testing approach, and is subject to the appreciable weaknesses described in Section 6.5.3. Notably, any increased variability within the test will weaken its power to distinguish toxic effects, resulting in an endpoint at a higher concentration (i.e., less toxicity is concluded). Similarly, use of only a few replicates instead of many replicates will weaken the discrimination of a test and will lead to a conclusion of less apparent toxicity, other things being equal (see Section 5.6.2). There is no alternative to hypothesis-testing for analysis of most toxicity tests with sediment, because they use only one concentration of samples, usually full-strength. There are superior alternatives for point estimates of toxicity if various concentrations are tested (see Section 6).

The parametric analyses with ANOVA and multiple-comparison tests assume that the data are normally distributed, and that the variance is homogeneous among the different groups. As the first step in analysis, these assumptions should be tested with *Shapiro-Wilk's Test* for normality and *Bartlett's Test for Homogeneity of Variance* (Eisenhart *et al.*, 1947; Sokal and Rohlf, 1969). If the data are satisfactory by these tests, analysis may proceed. If not, data could be transformed (e.g., as square roots, logarithms, or as arcsine square root for quantal data which are to be used in quantitative analysis; Mearns *et al.*, 1986). The tests for normality and homogeneity might then show conformance with normality

and homogeneity, and in fact that is a likely outcome of transformation.

Parametric tests are actually quite robust in the face of moderate deviations from normality and equality of variance. Therefore, parametric analysis (e.g., ANOVA and multiple comparison) should proceed, even if moderate nonconformity continues after transformation. Excluding a data-set for minor irregularities might lose a satisfactory and sensitive analysis and forgo the detection of real effects of toxicity.<sup>43</sup> Analysis by nonparametric tests should also proceed in parallel, with the more sensitive (lower endpoint) of the two analyses providing the final estimates of toxicity (see further explanation and rationale in Environment Canada, 1997d).

Multi-concentration tests might be conducted with sediment, sludge, or similar particulate material. Measured amounts of the test sample could be mixed with measured quantities of natural or formulated control sediment (see Sections 3.5 and 6.2). Procedures for mixing different samples of sediment are not yet standardized or proven (see Section 6.2), and caution is advised due to possible nonlinear responses and changes in bioavailability or sorption characteristics

---

<sup>43</sup> Tests for normality and homogeneity become less meaningful with the small samples of environmental toxicology. Plotting and examining the general nature of the distribution and apparent deviations can be more revealing and is recommended (EC, 1997d). Equality in sample sizes and the magnitude of variation are probably more important factors for the outcome of parametric analysis, but they have received scant attention in toxicology. Robustness of ANOVA is shown by its ability to produce realistic probabilities if the distribution of data is reasonably symmetrical, and if treatment variances are within threefold of each other (Newman, 1995).



(Nelson *et al.*, 1994). A minimum of five subsamples are recommended to provide replicates in each concentration, to determine sample homogeneity and test precision. Statistical analyses to determine endpoints are described in Section 6.5.

#### 5.6.1 Variations in Design and Analysis

A very preliminary survey might have only one test sample and one reference sample, without replication. Simple inspection of the results might provide guidance for designing more extensive studies.

If there were a single test sample and a reference sample, with equal replication for each, a standard *Student's t-test* would be suitable for analysis (Paine and McPherson, 1991). The *t-test* is fairly robust. The full formula for the *t-test* should be used, because it contains provisions for irregular data. The formula handles unequal numbers of replicates in the test and reference samples, as well as unequal variances in the two groups (USEPA/USACE, 1994). If there were *laboratory replicates* only, the interpretation of findings would be different from a survey in which there were *field replicates*, as described in the following for analysis of variance.

A preliminary study might conceivably be run with samples from many stations, but without either field replicates or laboratory (within-sample) replicates. The objective might be to identify a reduced number of sampling stations deserving of more detailed and further study. Opportunities for statistical analysis would be limited. The nonreplicated test data could be compared with the reference data using outlier detection methods (USEPA, 1994a; Newman, 1995; EC, 1997d). A sample

would be considered toxic if its result was rejected as an extreme value when considered as part of the reference and/or control data.

A more usual survey of sediments would involve the collection of samples from several places by the same methods, and their comparison with a single reference and/or control. There are several pathways for analysis, depending on the type and quality of data, but often there would be an *analysis of variance (ANOVA)* followed by one of the multiple-comparison tests. In the ANOVA, the reference would also be treated as a "location".

In these multi-location surveys, the type of replication would make a difference in how the results were interpreted. There might be one sample at each of a number of locations, with *laboratory replicates* obtained by subdividing each sample. In that case, the one-way ANOVA would distinguish whether there was an overall difference among locations, a difference that was greater than the baseline variability in the *within-laboratory* procedures for setting up and running the test. Sampling variability would not really be assessed in the statistical analysis, except that it would contribute to any difference found in locations. If *field replicates* were collected at each of the sampling locations, and no laboratory replicates were used, the same type of one-way ANOVA would evaluate overall difference in locations, over and above the *combined* variability of sampling the location and running the test. It would be unusual but much more powerful, to have field replicates for all sampling locations, and also laboratory replicates of each field replicate. If that were done, the laboratory replicates

would become the replicates in a nested one-way ANOVA, and would be the base of variability for comparing differences in the samples. The ANOVA could be used to see (a) if there was an overall difference in locations, and (b) whether there was an overall difference in replicates taken at the various locations. After an ANOVA, the analysis would proceed to one or more multiple-comparison tests, as described herein.

After the ANOVA for multi-location surveys, different statistical tests are available for application. If it were desired to compare each sampling location with the reference to see if the two were different, *Dunnnett's test* should be used. It assumes normality and equal variance, and is based on an *experiment-wise* value of  $\alpha$  (the probability of declaring a significant difference when none actually exists). If replication were unequal, investigators should seek the advice of a statistician, or adopt the complete interlocation comparison described in the following paragraph.

In a multi-location survey, an investigator might wish to know which sampling locations were different from which others, as well as knowing which ones were different from the reference and/or control. Such a situation might involve a number of locations "downstream" of an effluent discharge and one might want to know which locations had significantly higher toxicity than others, and were particularly deserving of clean-up. *Tukey's test* is designed for such an analysis, and is commonly found in statistics

packages. It can deal with unequal sample sizes.<sup>44</sup>

If it were desired to compare each sampling location with the reference, but the data did not conform to requirements of normality and equal variance, the ANOVA and subsequent tests would be replaced by non-parametric tests. *Steel's Many-One Rank test* would be used if replication were equal, while unequal replication would require *Wilcoxon Rank Sum test with Bonferroni's adjustment*.

### 5.6.2 Power Analysis

An important factor to consider in the analysis of toxicity tests with sediment is the potential for declaring false "positives" (i.e., calling a clean site dirty) or false "negatives" (i.e., calling a dirty site clean). Scientists are usually cautious in choosing the level of significance ( $\alpha$ ) for tolerating false positive

---

<sup>44</sup> An alternative approach is currently recommended for sediment testing in the United States (USEPA, 1994a; USEPA/USACE, 1994). For equal replicates, *Fisher's Least Significant Difference (LSD)* is said to be a superior approach. It is based on a smaller "pairwise error rate" for  $\alpha$  in comparing any given location with another, but holds the overall value of  $\alpha$  to the pre-selected value (usually 0.05). *LSD* is seldom included in software packages for toxicity, but it is described in some textbooks (e.g., Steel and Torrie, 1980), and is detailed in Appendix D of USEPA/USACE (1994). *Tukey's test* is instead recommended here, partly because *LSD* might declare significant differences too readily. *LSD* is also intended for only a few of all the possible comparisons in a set of data, and those comparisons would have to be specified in advance. In the current U.S. recommendations for pairwise comparison, a situation with unequal replicates would call for *Bonferroni's adjustment of the t-test* to replace testing by *LSD* (USEPA, 1994a). That adjusted t-test enables all possible comparisons among locations.

results (Type I error), and usually set it at  $P = 0.05$  or  $0.01$ . Recently, toxicologists have been urged to report both  $\alpha$  and statistical power ( $1 - \beta$ ), i.e., the probability of correctly rejecting the null hypothesis ( $H_0$ ) and not making a Type II error. There are several factors that influence statistical power, including:

- variability of replicate samples representing the same treatment;
- $\alpha$  (i.e., the probability of making a Type I error);
- effect size ( $ES$ ) (i.e., the magnitude of the true effect for which you are testing); and

- $n$  (i.e., the number of samples or replicates used in a test).

Power analysis can be used *a priori* to determine the magnitude of the Type II error and the probability of false positive results. It can also be used to ascertain the appropriate number of field and laboratory replicates for subsequent surveys involving this test, or to assist in the selection of future sampling sites. It is always prudent to include as many replicates in the test design as is economically and logistically warranted (see Section 5.1); power analysis will assist in this determination. A good explanation of the power of a test, and how to assess it, can be found in USEPA (1994a), with some coverage in EC (1997d).

## Section 6

---

### Specific Procedures for Testing Spiked Sediment

This section gives guidance and instructions for preparing and testing control or other sediment spiked experimentally with chemical(s), contaminated sediment, or complex waste mixtures. These recommendations and instructions are in addition to the procedures listed in Section 4. More detailed and appropriate guidance for spiking sediment with chemical(s) and conducting toxicity tests with chemical/sediment mixtures is given in Environment Canada (1995). Depending on the test objectives or constraints, and on any related regulatory guidelines or requirements, these toxicity tests can be conducted using either the daily-renewal test option or the static test option (see Section 4). Further evaluation and standardization of procedures for spiking sediment (Section 6.2) might be required before sediment toxicity tests using *Chironomus* sp. or other appropriate test organisms are applied to evaluate specific chemical/sediment mixtures for regulatory purposes.

The cause(s) of sediment toxicity and the interactive toxic effects of chemical(s), contaminated sediment, or particulate waste in association with otherwise clean sediment can be examined experimentally by spiking *clean* control sediment with these substances or materials. The spiking might be done with one or more chemicals, with another sediment (clean or uncontaminated), or with similar particulate material (e.g., dredged sludge mixed with sediment from an existing or prospective freshwater disposal site). Toxicity tests using sediment spiked with a range of concentrations can estimate LC50s,

and can determine concentrations causing sublethal effects. The influence of the physicochemical characteristics of natural or formulated sediment on chemical toxicity can also be determined with spiked-sediment toxicity tests. Reference toxicity tests can also be conducted using control sediment spiked with an appropriate chemical (see Section 4.8). Specific recommendations and instructions for performing spiked-sediment tests are provided in this section. (Additional useful guidance is given in USEPA, 1994a and EC, 1995.)

#### 6.1 *Sample Properties, Labelling, and Storage*

Information should be obtained on the properties of the chemical, contaminated sediment, or particulate waste to be diluted experimentally with control or other sediment. For samples of contaminated sediment or similar particulate material, instructions on sample characterization (Section 5.2) should be followed. For individual chemicals, chemical substances (e.g., formulated products), or chemical mixtures, available information should be obtained on the concentration of major ingredients and impurities, water solubility, vapour pressure, chemical stability, dissociation constants, toxicity to humans and aquatic organisms, and biodegradability. Where aqueous solubility is in doubt or problematic, acceptable procedures previously used for preparing aqueous solutions of the chemical(s) should be obtained and reported, and chemical

solubility in test water should be determined experimentally. Other available information such as structural formulae, nature and percentage of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient, should be obtained and recorded.

Chemical(s) to be tested should be at least reagent grade, unless a test on a formulated commercial product or technical grade chemical(s) is required (USEPA, 1994a). Chemical containers must be sealed and coded or labelled upon receipt. Required information (chemical name, supplier, date received, person responsible for testing, etc.) should be indicated on the label and/or recorded on a separate datasheet dedicated to the sample, as appropriate. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Sample(s) of contaminated sediment or particulate waste to be evaluated in spiked-sediment assays should be collected, labelled, transported, and stored according to instructions herein (Sections 5.1 and 5.2).

## 6.2 Preparing Test Mixtures

Different procedures have been used by researchers to spike (dose) *clean* control sediment with chemical(s), or to dilute control sediment with contaminated sediment or other particulate waste with control sediment, in preparation for sediment toxicity tests with the mixture (ASTM, 1991a; b; 1994; 1995a; b; Burton, 1991; USEPA, 1994a; Hoke *et al.*, 1995). Mixing technique and time, as well as the period of aging after mixing, can affect the toxicity of the mixture (USEPA, 1994a).

Experimental procedures (including substance addition and mixing, equilibration time and conditions) used to prepare spiked sediment are new, varied, and not standardized. Accordingly, a standardized methodology for preparing spiked sediment cannot be recommended at this time. Rather, some of the approaches used previously or thought to be reasonable for preparing spiked sediment for toxicity tests with *Chironomus* sp. are given here.

Environment Canada (1994; 1995) reports provide more detailed instructions and recommendations for spiking and homogenizing sediment, and should be consulted for further guidance. Researchers intending to pursue toxicity tests using one or more laboratory-prepared mixtures should proceed cautiously, and should be well aware of potential problems due to nonhomogeneity of the mixture(s) and the associated changes in bioavailability/sorption characteristics and nonlinear toxic responses that might result (Nelson *et al.*, 1994).

The method to be used is contingent on the study objectives and the nature of the test substance or material to be mixed with control or other sediment. In many instances, a chemical/sediment mixture is prepared by making up a stock solution of the chemical and then mixing one or more measured volumes into control sediment (ASTM, 1991a, 1994; USEPA, 1994a). Chemical concentrations in sediment are frequently calculated and expressed as  $\mu\text{g/g}$  or  $\text{mg/kg}$  dry weight, although concentrations based on wet weight might be more useful for relating results to sediment toxicity (Burton, 1991). Depending on the nature of the test substance or material and



test objectives, concentrations might also be normalized to sediment organic carbon content (e.g., for evaluating the toxicity of nonpolar organic compounds) or to acid volatile sulphides (e.g., for assessing metal toxicity) (Di Toro *et al.*, 1990; 1991; ASTM, 1991a; 1994; USEPA, 1994a).

The preferred solvent for preparing stock solutions is test water (see Sections 2.3.4 and 3.4); use of a solvent other than water should be avoided unless it is absolutely necessary. For organic compounds or other chemicals that do not dissolve readily in test water, a water-miscible organic solvent may be used to help disperse the compound in water (ASTM, 1991a; 1994; USEPA, 1994a). Triethylene glycol has been recommended because of its low toxicity to aquatic organisms, low volatility, and high ability to dissolve many organic chemicals (ASTM, 1991a; 1994). Other solvents such as methanol, ethanol, dimethylsulphoxide, or acetone may be used to prepare stock solutions of organic chemicals, although they might contribute to sample toxicity, alter sediment properties, or be lost from the test material due to their volatility. Surfactants should not be used (ASTM, 1991a; 1994).

If an organic solvent is used, the test must be conducted using both a clean sediment control (i.e., no solvent and no test substance) and a sediment control containing solvent. For this purpose, a *solvent control* sediment must be prepared containing the concentration of solubilizing agent that is present in the highest concentration of the test chemical in sediment. Solvent from the same batch used to make the stock solution must be used (ASTM, 1991a; 1994; USEPA, 1994a).

Solvents should be used sparingly as they might contribute to the toxicity of the prepared test sediment. The maximum concentration of solvent in the sediment should be at a concentration that does not affect the survival or growth of midge larvae during the test. If this information is unknown, a preliminary *solvent only* test, using various concentrations of solvent in control sediment, should be conducted to determine the threshold-effect concentration of the particular solvent being considered for use in the definitive test.

Measured volumes of a stock solution containing test chemical(s) should be mixed with control (or other) sediment in a manner resulting in a homogeneous distribution of the chemical(s) throughout the sediment. Mixing may be by hand (e.g., using a clean spatula or glass rod) or by using a mechanical stirring or mixing device (e.g., Ditsworth *et al.*, 1990). Alternatively, the chemical can be coated on the walls of a flask and an aqueous slurry (i.e., control sediment and test water) added. The flask contents are then mixed by agitation. Another alternative is to add a measured volume of the stock chemical solution directly to a slurry of control (or other) sediment in test water, agitate the mixture, and allow it to settle (EC, 1992a). Other methods of mixing might prove to be acceptable provided that the chemical is shown to be evenly distributed in the sediment. Mixing conditions, including solution:sediment ratio, mixing and holding time, and mixing and holding temperature, must be standardized for each treatment included in a test. Time for mixing a spiked sediment should be adequate to ensure homogeneous distribution of the chemical, and may be from minutes up to 24 hours.

During mixing, temperature should be kept low to minimize changes in the mixture's physicochemical characteristics and microbial activity. Analyses of subsamples of the mixture are advisable to determine the degree of mixing and homogeneity (Ditsworth *et al.*, 1990; USEPA, 1994a).

For some studies, it might be necessary to prepare only one concentration of a particular mixture of control (or other) sediment and chemical(s), or a mixture of only one concentration of contaminated sediment or particulate waste in control or other sediment. For instance, a single-concentration test might be conducted to determine whether a specific concentration of chemical or particulate waste in clean sediment is toxic to *C. tentans* or *C. riparius*. Such an application could be used for research or regulatory purposes.

A multi-concentration test, using a range of concentrations of chemical added to a control or other sediment under standardized conditions, should be used to determine the endpoint (e.g., LC50, ICp, NOEC, LOEC; see Section 6.5) for chemical/sediment mixtures. A multi-concentration test using control sediment spiked with a specific particulate waste might also be appropriate. For such purposes, at least five test concentrations plus a control must be prepared; and the preparation and use of six to eight concentrations (plus one or more control sediments) is recommended to improve the likelihood of attaining each endpoint sought. An appropriate geometric dilution series may be used, in which each successive concentration of chemical or particulate waste in sediment is at least 50% of the previous (e.g., 10, 5, 2.5, 1.25,

0.63 mg/kg).<sup>45</sup> Test concentrations may also be selected from other appropriate logarithmic dilution series (see Appendix H). To select a suitable range of concentrations, a preliminary or range-finding test covering a broader range of test concentrations may be conducted.

Tests intended to evaluate the toxicity of mixtures of test substance(s) or material(s) in control sediment for federal registration or other regulatory purposes must be set up using a minimum of five replicates for each test concentration and each control sediment to be included in the assay. Since the objective for a multi-concentration test is to determine both LC50 (mortality data) and ICp (dry weight data), a test using six to eight concentrations plus control(s) is recommended. The number of replicates per treatment could be reduced or eliminated altogether for range-finding tests and, depending on the expected variance among test chambers within a treatment, could also be reduced or eliminated for nonregulatory screening assays or research studies.

It is recommended that mixtures of spiked sediment be aged for four weeks before starting a test, in keeping with a common practice (USEPA, 1994a). Although many studies with spiked sediment have been started within a few hours or days of preparing the mixtures, such short and variable time periods might not be long enough for equilibration of the chemicals mixed in control sediment. A consistent four-week period of aging a mixture before

---

<sup>45</sup> Concentrations in sediment are normally calculated and expressed as  $\mu\text{g/g}$  or  $\text{mg/kg}$ , on a dry-weight or wet-weight basis. In some instances, concentrations in pore water might also be measured and expressed as  $\mu\text{g/L}$  or  $\text{mg/L}$ .

initiating a toxicity test would provide some standardization for intra- and interlaboratory comparisons of results for tests with spiked sediment. Once prepared, each mixture should be placed in a suitable, sealed (with no air space) container, and stored in the dark at  $4 \pm 2^\circ\text{C}$  (Section 5.2) for four weeks before use.

Based on the objectives of the test, it might be desirable to determine the effect of substrate characteristics (e.g., particle size or organic content) on the toxicity of chemical/sediment mixtures. For instance, the influence of sediment particle size on chemical toxicity could be measured by conducting concurrent multi-concentration tests with a series of mixtures comprised of the test chemical mixed in differing fractions (i.e., segregated particle sizes) or types of natural or formulated control sediment (Section 3.5). Similarly, the degree to which the organic content of sediment can modify chemical toxicity could be examined by performing concurrent multi-concentration tests using with a different chemical/sediment mixtures prepared series of organically enriched control sediments. Each fraction or formulation of natural or artificial control sediment used to prepare such mixtures should be included as a control in the test.

Tests could be required to measure the effect on survival or weight of *Chironomus* sp. at test end, for one or more concentrations of specific chemicals introduced to the test chamber as a solution overlying the sediment. Procedures for preparing test concentrations could vary depending on the objectives of the study. One approach would be to carefully add the test solution(s) to replicate chambers containing a layer of

control or other (e.g., field-collected) sediment, with no disturbance or subsequent mixing of the sediment and test solution(s). A second approach would require the test solution(s) introduced to test chambers to be agitated for a predetermined time period in the presence of the sediment before the test organisms are introduced.

Chemical/sediment interactions might differ appreciably depending on the approach taken, and could result in a markedly different test result. Unless specified or otherwise required, test water adjusted to  $23 \pm 1^\circ\text{C}$  should be used to prepare each test solution (Section 6.3). Replicate controls, including solvent controls if a solvent is used, must be prepared and treated identically. Instructions provided earlier in this section on the use of solvents other than water should be followed in preparing solvent controls.

### 6.3 Test and Control/Dilution Water

The water used for preparing stock or test solutions of chemicals and as test water in 10-day assays with mixtures of spiked sediment should normally be clean test water (see Section 3.4). The source of this water may be reconstituted water or natural water, and might or might not be identical to the water used for culturing the test organisms (see Section 2.3.4). Reconstituted water with a hardness of 90 to 100 mg  $\text{CaCO}_3/\text{L}$  (Section 2.3.4; USEPA, 1994a) is recommended if a high degree of standardization is required. For example, the use of a standard reconstituted water is recommended in instances where the measured toxicity of the chemical/sediment mixture is to be compared and assessed

relative to toxicity data derived at a number of test facilities for this and/or other chemicals.

#### **6.4 Test Observations and Measurements**

A qualitative description of each mixture of spiked sediment and of the overlying test water should be made when the test is being established. This might include observations of the colour, texture, and visual homogeneity of each mixture of spiked sediment, and observations of the colour and opacity of the overlying water. Any change in appearance of the test mixture or overlying water noted during the test, or upon its termination, should be recorded. Measurements of the quality of each mixture of spiked sediment being tested (including the control sediment), and of the overlying water, should be made and recorded as described in Sections 4.5, 5.2, and 5.5.

If analytical capabilities permit, it is recommended that stock solutions, overlying water, whole sediment, pore water, and test solutions (if studied) be analyzed to determine the chemical concentrations, and to assess whether the sediment has been spiked satisfactorily. In instances where chemical concentrations are to be measured, sample aliquots should be taken from the high, medium, and low test concentrations at the beginning and end of the test, as a minimum. These should be preserved, stored, and analyzed according to suitable, validated procedures.

Unless there is good reason to believe that the chemical measurements are not accurate, toxicity results for any tests in which

concentrations are measured should be calculated and expressed in terms of those average measured concentrations determined for both the whole sediment ( $\mu\text{g/kg}$  or  $\text{mg/kg}$ , dry weight) and the pore water ( $\mu\text{g/L}$  or  $\text{mg/L}$ ). In cases where concentrations of chemical added to the overlying water are being tested, results should again be expressed as the average measured concentrations determined for the whole sediment and the pore water, although average chemical concentrations measured for the test solutions overlying sediment should also be calculated and reported (EC, 1992a; 1997a; b).

#### **6.5 Test Endpoints and Calculations**

Multi-concentration tests with mixtures of spiked sediment are characterized by the 10-day  $\text{LC}_{50}$  and an endpoint representing the weight data (e.g.,  $\text{ICp}$ ). Appropriate statistics and programs for calculating these endpoints are summarized in this section. Section 5.6 provides guidance for calculating and comparing endpoints for single-concentration tests performed with mixtures of spiked sediment. For further information on the appropriate parametric or nonparametric statistics to apply to the endpoint data, the investigator should consult the Environment Canada report on statistics for the determination of toxicity endpoints (EC, 1997d), as well as USEPA (1994a; b) or USEPA/USACE (1994).

##### **6.5.1 Median Lethal Concentration ( $\text{LC}_{50}$ )**

When a multi-concentration test with spiked-sediment mixtures is conducted (Section 6.2), the quantal mortality data must be used to calculate the 10-day *median lethal*



concentration (LC50), together with its 95% confidence limits. To estimate an LC50, mortality data at 10 days are combined for all replicates at each concentration. If mortality is not  $\geq 50\%$  in at least one concentration, the LC50 cannot be estimated. If there are no mortalities at a specific concentration, that information is used as an effect of 0% mortality. However, if successive concentrations yield a series of 0% mortalities, only the highest concentration of the series should be used in estimating the LC50 (i.e., the zero-effect that is "closest to the middle" of the distribution of data). Similarly, if there were a series of successive complete mortalities at the high concentrations in the test, only one value of 100% effect would be used, i.e., the one at the lowest concentration. Use of only one 0% and one 100% effect applies to any form of statistical analysis and to hand plotting on a graph.

Various computer programs may be used to calculate the LC50. Stephan (1977) developed a program to estimate LC50s using probit, moving average, and binomial methods, and adapted it for the IBM-compatible personal computer. This program in the BASIC language is recommended, and is available on diskette<sup>46</sup> from Environment Canada (address in Appendix B). Other satisfactory computer and manual methods may be used (e.g., USEPA, 1985a; Hubert, 1987; APHA *et al.*, 1995; EC, 1997d). Programs using the trimmed Spearman-Kärber method (Hamilton *et al.*, 1977) are available for personal computers but are not recommended because divergent results

might be obtained by operators who are unfamiliar with the implications of trimming ends of the dose-response data (EC, 1997d).

The recommended program of Stephan (1977) provides estimates of LC50 and confidence limits by each of its three methods if there are at least two partial mortalities in the set of data. For smooth or regular data, the three results will likely be similar, and values from the probit analysis should be taken as the preferred ones and reported. The probit analysis also gives the slope of the line, which should be reported. The binomial estimate might differ somewhat from the others, and this estimate should only be used as a last resort. If the results do not include two partial mortalities, only the binomial method can be used to provide an estimate of the LC50. Formal confidence limits are not estimated using the binomial method; instead, outer limits of a range are provided, within which the LC50 and the true confidence limits would lie.

Any computer-derived LC50 should be checked by examining a plot, on logarithmic-probability scales, of percent mortalities at Day 10 for the various test concentrations (APHA *et al.*, 1995; EC, 1997d). Any major disparity between the estimated LC50 derived from this plot and the computer-derived LC50 must be resolved. A hand-plotted graph is preferred for this check. A computer-generated plot could be used if it were based on logarithmic-probability scales. If there had been an error in entering the data, however, a computer-generated plot would contain the same error as the mathematical analysis, and so the investigator should carefully check for correct placement of points.

<sup>46</sup> Through the courtesy of Dr. Charles E. Stephan (USEPA, Duluth, MN).



A manual plot of mortality/concentration data to derive an estimated LC50 is illustrated in Figure 2. In this hypothetical example, there were 100 animals (five replicates of 20 organisms each, per concentration) tested at each of five concentrations. This figure was based on concentrations of 1.8, 3.2, 5.6, 10, and 18 mg chemical/kg sediment causing mortalities of 0, 20, 40, 90, and 100% for the groups of midge larvae exposed to the respective concentrations for 10 days. The concentration expected to be lethal to 50% of the organisms can be read by following across from 50% (broken line) to the intersection with the best-fit line, then down to the horizontal axis for an estimated LC50 (5.6 mg/kg). A similar plot could be made of mortality/concentration data using the average measured concentration (in  $\mu\text{g/L}$  or  $\text{mg/L}$ ) determined for the porewater analyses (see Section 6.4).

In fitting a line such as that in Figure 2, relatively more emphasis should be assigned to points that are near 50% mortality. Logarithmic-probability paper (*log-probit*, as in Figure 2) can be purchased in good technical bookstores, ordered through them, or photocopied (see blank graph in EC, 1997d).

For the regular set of data in Figure 2, computer programs gave very similar estimates to the graphic one. The LC50s (and 95% confidence limits) were:

Stephan (1977) method:

- probit: 5.58 (4.24 and 7.37)
- moving average: 5.58 (4.24 and 7.33)
- binomial: 6.22 (between 1.8 and 10)

Probit analysis  
of Hubert (1987): 5.56 (4.28 and 7.21)

TOXSTAT 3.4: 5.58 (4.38 and 7.12)

SAS (1988)  
probit analysis: 5.58 (4.26 and 7.40)

If a solvent control is included in the study, the dry weight of test organisms in replicates of the *solvent control* sediment should be compared statistically with weights from the *clean control* sediment. Student's *t*-test may be applied for this comparison (Section 5.6.1). If the weights for the two controls differ significantly, only the solvent control may be used as the basis for comparison and calculation of results. If the results are the same, the data from both controls should be used for assessing the acceptability of the test and as the basis for calculating results (USEPA, 1994a). The test is rendered invalid if more than 30% of the midge larvae held in either control sediment die during the test.

#### 6.5.2 Inhibiting Concentration for a Specified Percent Effect (ICp)

For the data on mean dry weight, the ICp (*inhibiting concentration for a specified percent effect*) is the recommended statistical endpoint. The ICp is a quantitative estimate of the concentration causing a fixed percent reduction in mean dry weight of test organisms (e.g., the IC25 and/or IC20, which represent 25% and 20% reduction). The desired value of *p* is selected by the investigator, and 25% or 20% is currently favoured. Any ICp that is calculated and reported must include the 95% confidence limits.

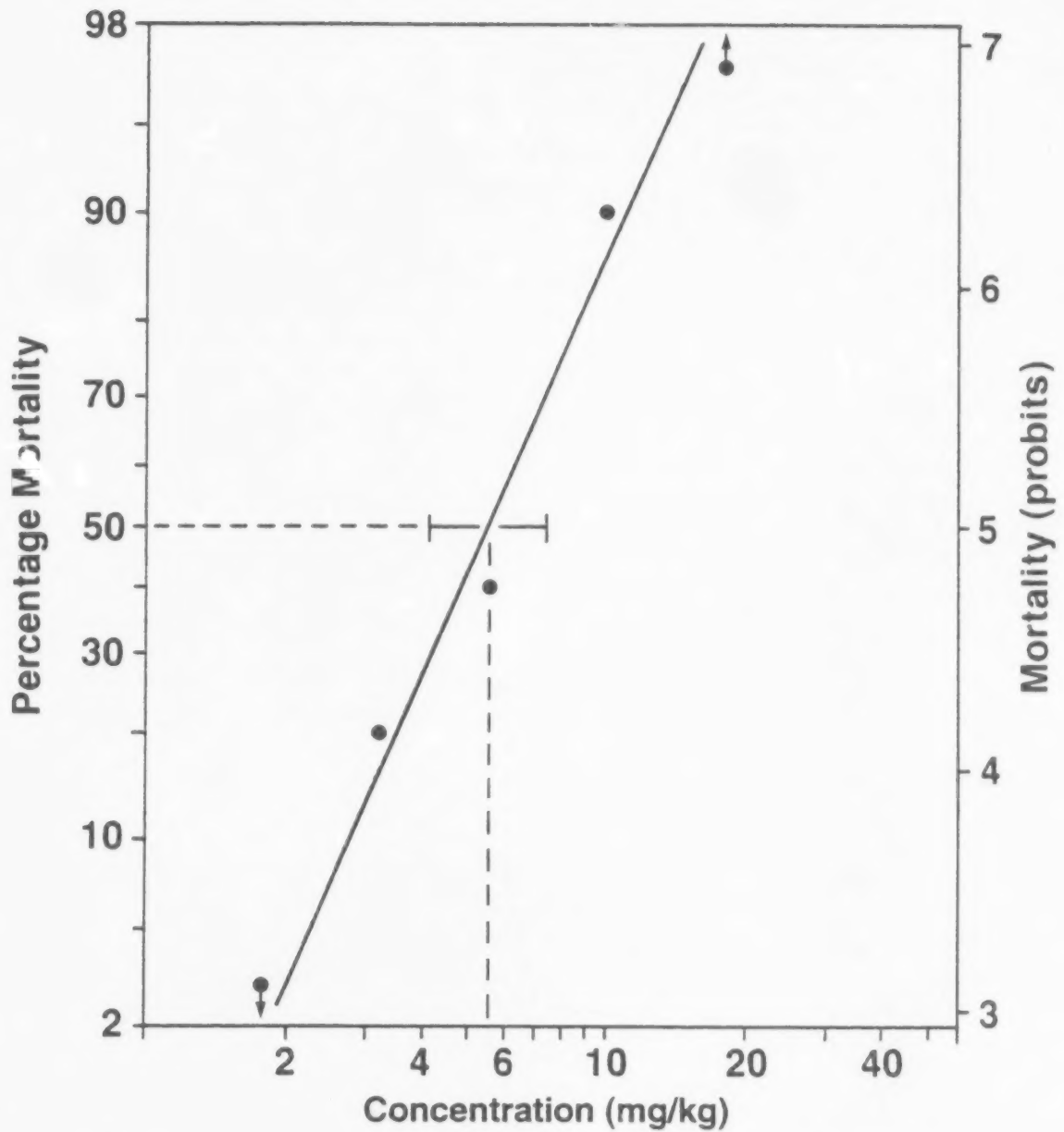


**Figure 2** Estimating a Median Lethal Concentration by Plotting Mortalities on Logarithmic-probability Paper

The mean weight of test organisms is calculated as the total dry weight of the midge larvae that survived in a given chamber, divided by the number of organisms that survived to the end of the test (Section 4.7). If there are no survivors in a replicate (chamber), that replicate is excluded from the analysis. If there is complete mortality in all replicates at a given concentration, that concentration is excluded.

At present, the only easily available method of estimating the ICp and its 95% confidence limits is the "bootstrap" method on computer (Norberg-King, 1993), a program called *ICPIN* (USEPA, 1994b). *ICPIN* is not proprietary, is available from USEPA, and is included in most computer software for environmental toxicology, including TOXSTAT. The original instructions for *ICPIN* from USEPA are clearly written and make the program easy to use (Norberg-King, 1993).<sup>47</sup> An earlier version was called *BOOTSTRP*.

---

<sup>47</sup> The instructions in Norberg-King (1993) are sometimes misleading on the identity of "replicates". The term is used in such a way that it would apply to weights of individual organisms within the same chamber. This slip of wording does not affect the functioning of the program.

Commercial packages available in 1996 were more difficult for entry of data and analysis, than the original program of USEPA (Norberg-King, 1993). Some packages were recalcitrant, with manuals that offered minimal guidance. Current commercial programs generally require and produce particular information specified by USEPA which would not necessarily satisfy requirements of Environment Canada. A commercial program might also be written for reasonably modern personal computers (1996+); one required enough conventional memory (at least 610 free kilobytes) that little might be left for other programs.

Analysis by *ICPIN* does not require equal numbers of replicates in different concentrations. The ICp is estimated by smoothing of the data as necessary, then using the two data-points adjacent to the selected ICp (USEPA, 1994b). The ICp cannot be calculated unless there are test concentrations both lower and higher than the ICp; both those concentrations should have an effect reasonably close to the selected value of p, preferably within 20% of it. At present, the computer program does not use a logarithmic scale of concentration, and so Canadian users of the program must enter the concentrations as logarithms. Some commercial computer packages have the logarithmic transformation as a general option, but investigators should make sure that it is actually retained when proceeding to *ICPIN*. *ICPIN* estimates confidence limits by a special "bootstrap" technique because usual methods would not be valid. Bootstrapping performs many resamplings from the original measurements. The investigator must specify the number of resamplings; it can range from 80 to 1000, at least 400 is recommended herein, and 1000 would be beneficial.<sup>48</sup>

---

<sup>48</sup> *ICPIN* has some deficiencies. Its interpolation method is an inefficient use of data, sensitive to peculiarities of the two concentrations used. The program fails to adopt logarithm of concentration, which would introduce a slight bias towards a higher value of ICp. A modification of the bootstrap method has now remedied a problem of overly narrow confidence limits. Linear regression or general-purpose regression would be better methods of estimating the ICp and its 95% confidence limits (EC, 1997d), but a standard "packaged" method of regression has not been developed for environmental toxicology. Investigators should watch for any development of such a suitable new program.

Besides determining and reporting a computer-derived IC<sub>p</sub>, a graph of percent reduction of dry weight against the logarithm of concentration should be plotted, to check the mathematical estimation and to provide a visual assessment of the nature of the data (EC, 1997d).

If a *solvent control* is included in the study, the weight of surviving midge larvae in its replicates should be compared statistically with weights from the control using clean sediment. *Student's t-test* may be applied for this comparison. If the weights differ significantly in the two controls, only the solvent control may be used as the basis for comparison and calculation of results. If the results are the same, the data from both controls should be used for assessing the acceptability of the test and as the basis for calculating results (USEPA, 1994a). The test is rendered invalid if more than 30% of the midge larvae held in either control sediment die during the test.

### 6.5.3 Hypothesis Testing (NOEC and LOEC)

An optional approach for presenting the results of the test is determining the *no-observed-effect concentration (NOEC)*, *lowest-observed-effect concentration (LOEC)*, and *threshold-observed-effect concentration (TOEC)*. For these spiked-sediment tests, NOECs and LOECs are calculated from the mean dry weights of surviving test organisms in each replicate (chamber) of the control and the various concentrations. Calculations use the same sublethal data as in estimating the IC<sub>p</sub>. If there is complete mortality in a replicate or a

concentration, it is excluded from the analysis.<sup>49</sup>

Statistical procedures are explained with some guidance in USEPA (1994a; b), USEPA/USACE (1994), Newman (1995), EC (1997d), and in commercial software packages such as TOXSTAT (WEST and Gulley, 1996). The methods start with a check of normality and homogeneity of variance by the *Shapiro-Wilks* and *Bartlett's tests*. If both tests are satisfied by the data in their original state or transformed (Section 5.6), analysis should proceed with parametric methods.

For parametric testing, an *analysis of variance (ANOVA)* is carried out, then *Williams' test*, a multiple-comparison test that determines which concentrations are significantly different from the control. Williams' test takes into account the order of concentrations by magnitude, a desirable feature to increase sensitivity, and a very appropriate attribute for most toxicity tests (Masters *et al.*, 1991).<sup>50</sup> Williams' test estimates the *Minimum Significant Difference (MSD)*. This is the magnitude of the difference in mean weights that would

<sup>49</sup> It is conceivable that significant mortality might occur at lower concentrations than those affecting mean weight. Although other methods of analysis might quantify such an effect on mortality, LC50 is the only endpoint for mortality that is to be reported for this sediment toxicity test.

<sup>50</sup> Another standard multiple-comparison test, *Dunnnett's test*, is given more prominence in TOXSTAT and most methods from the United States. It is not a particularly powerful way of discriminating effects since it ignores the magnitudes of the concentrations when it calculates the MSD (Masters *et al.*, 1991).

have to exist between the control and a test concentration, in order to conclude that there was a significant effect at that concentration. Any test which reports NOEC/LOEC must also report the MSD. If the MSD of average weights is >25% of the mean weight of the controls, the validity and usefulness of the findings is questionable.

If there are unequal numbers of replicates because of accidental loss or other cause, Williams' test is replaced by the *Dunn-Sidak modification of the t-test*, or by *Bonferroni's adjustment of the t-test*.

If tests for conformity and homogeneity cannot be satisfied by transformation, the parametric analysis should proceed except in cases of severe departure from normality which clearly would not fit such an analysis. The parametric tests are relatively robust in the face of moderate nonconformance (see Section 5.6). Nonparametric analysis should also proceed. The more sensitive (lower concentrations) of the two analyses is to be used as the final estimate of NOEC and LOEC. In this situation, detailed reporting must include the following items:

- results of the *Shapiro-Wilks* and *Bartlett's tests*;
- a hand-plotted graph of mean weights by chamber, using logarithmic concentration;
- findings of parametric analysis, including MSD; and
- findings of nonparametric analysis.

Nonparametric analysis requires four replicates.<sup>51</sup> *Shirley's test* would be the method of choice for use instead of analysis of variance. It parallels Williams' test in taking into consideration the ranking of concentration. Unfortunately, Shirley's test is not available in most statistical packages, nor is it described in most textbooks. *Steel's many-one rank test* is offered in most U.S. statistical packages, and could be used in this situation; it does not consider the order of concentrations. If there were unequal replication, the *Wilcoxon rank sum test* should be used. These tests are strong tools for data that are not normally distributed, but they would be less powerful than parametric tests if used on normally distributed data.

The geometric mean of the NOEC and LOEC, the *TOEC*, is often calculated for the convenience of having one number. The TOEC should be reported, recognizing that it is an arbitrary estimate of an effect-threshold that might lie anywhere in the range between the LOEC and NOEC. Its value is governed by whatever concentrations were selected for the test. No confidence limits can be estimated for the NOEC, LOEC, or TOEC.

---

<sup>51</sup> The requirement for four replicates might prevent an estimation of NOEC/LOEC. A test might have been designed with fewer replicates, primarily for calculating the ICp. If results were found to deviate from normality or homogeneity, the investigator would not be able to complete the analysis by nonparametric methods.



## Section 7

---

### Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the *must* requirements delineated in Sections 2 to 6 of this biological test method, and, if so, provide details of deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 7.1 provides a list of items that must be included in each test-specific report. A list of items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years, is found in Section 7.2. Specific monitoring programs, related test protocols, or regulations might require selected test-specific items listed in Section 7.2 (e.g., details about the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as *data to be held on file*.

Procedures and conditions common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes) and consistent with specifications in this document, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice

Details on the conduct and findings of the test, which are not conveyed by the test-

specific report or general report, must be kept on file by the laboratory for a minimum of five years so that the appropriate information can be provided if an audit of the test is required. Filed information might include:

- a record of the chain-of-continuity for field-collected or other samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- chemical analytical data on the sample(s) not included in the test-specific report;
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source of the test organisms, their taxonomic confirmation, and all pertinent information regarding their culturing and health; and
- information on the calibration of equipment and instruments

Original data sheets must be signed or initialed, and dated by the laboratory personnel conducting the tests

## **7.1 Minimum Requirements for a Test-specific Report**

Following is a list of items that must be included in each test-specific report.

### **7.1.1 Test Substance or Material**

- brief description of sample type (e.g., dredged material, reference or contaminated field-collected sediment, control sediment), or coding, as provided to the laboratory personnel;
- information on labelling or coding of each sample; and
- date of sample collection; date and time sample(s) received at test facility.

### **7.1.2 Test Organisms**

- species and source of brood stock and test organisms;
- instar, at start of test;
- for *C. tentans* only, mean and range of head capsule width at start of test; and
- any unusual appearance or treatment of the organisms, before their use in the test.

### **7.1.3 Test Facilities**

- name and address of test laboratory; and

- name of person(s) performing the test.

### **7.1.4 Test Water**

- type and source of test water; and
- measured characteristics of test water, before and/or at time of commencement of toxicity test.

### **7.1.5 Test Method**

- citation of biological test method used (i.e., as per this document);
- design and description if specialized procedure (e.g., sieving of field-collected test sediment; preparation of mixtures of spiked sediment; preparation and use of solvent and, if so, solvent control) or modification of standard test method;
- brief description of frequency and type of observations and measurements made during test; and
- name and citation of program(s) and methods used for calculating statistical endpoints.

### **7.1.6 Test Conditions and Procedures**

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- number of discrete samples per treatment; number of replicate test

chambers for each treatment; number and description of treatments in each test including the control(s); test concentrations (if applicable);

- depth and volume of sediment and overlying water in each test chamber;
- number of organisms per test chamber and treatment;
- test option chosen (i.e., daily-renewal or static);
- feeding regime and ration;
- indication if any aeration of overlying water (including rate);
- dates when test was started and ended;
- for each sample — all measurements of sediment particle size, percent water content, and total organic carbon; and of porewater pH and ammonia; and
- for at least one test chamber representing each treatment — all measurements of temperature and dissolved oxygen in overlying water, made at start of test and three or more times per week thereafter; all measurements of conductivity, pH, and ammonia in overlying water, made at start and end of test.

#### 7.1.7 *Test Results*

- for each treatment — mean  $\pm$  SD for percentage of midge larvae that survived the 10-day exposure; mean

$\pm$  SD for dry weight of surviving amphipods at test end; results of any statistical comparisons;

- coefficient of variation (CV) for mean percent survival and mean individual dry weight of replicate control groups at test end;
- any LC50 (including the associated 95% confidence limits and, if calculated, the slope) determined;
- any ICp (together with its 95% confidence limits) determined for the growth (i.e., dry weight at test end); details regarding any transformation of data that was required, and indication of quantitative statistic used;
- for a multi-concentration test with spiked sediment, indication as to whether results are based on nominal or measured concentrations of a particular substance or material;
- results for any 96-h LC50 (including its 95% confidence limits) performed with the reference toxicant(s) using the same batch of test organisms, together with the geometric mean value ( $\pm$  2 SD) for the same reference toxicant(s) and test species as derived at the test facility in previous tests using the procedures and conditions herein; and
- anything unusual about the test, any problems encountered, any remedial measures taken.

## **7.2 Additional Reporting Requirements**

Following is a list of items that must be either included in the test-specific report or the general report, or held on file for a minimum of five years.

### **7.2.1 Test Substance or Material**

- identification of person(s) who collected and/or provided the sample;
- records of sample chain-of-continuity and log-entry sheets; and
- conditions (e.g., temperature, in darkness, in sealed container) of sample upon receipt and during storage).

### **7.2.2 Test Organisms**

- name of person(s) who identified the organisms and the taxonomic guidelines used to confirm species;
- history and age of brood stock;
- description of culture conditions and procedures (e.g., facilities and apparatus, lighting, water source and quality, water pretreatment, water exchange rate and method, water temperature, type and quantity of substrate) for cultures;
- procedures used to count, handle, sort, transfer, and sieve animals; and those to determine their mortality, condition, appearance, and behaviour; and

- source and composition of food, procedures used to prepare and store food, feeding method(s), feeding frequency and ration.

### **7.2.3 Test Facilities and Apparatus**

- description of laboratory's previous experience with this biological test method for measuring sediment toxicity using midge larvae;
- description of systems for providing lighting and compressed air, and for regulating temperature within test facility;
- description of test chambers, and covers if used;
- description of apparatus used to deliver and renew overlying water in test chambers, if the daily-renewal test option is chosen; and
- description of procedures used to clean or rinse test apparatus.

### **7.2.4 Control Sediment and Test Water**

- procedures for pretreatment of control sediment (e.g., sieving, settling of sieved fines, formulation and aging if formulated) and test water (e.g., filtration, sterilization, reconstitution and aging if reconstituted, temperature adjustment, aeration rate and duration);
- type and quantity of any chemical(s) added to test water; and

- storage conditions and duration before use.

#### 7.2.5 *Test Method*

- procedures used for mixing or otherwise manipulating test sediments before use; time interval between preparation and testing;
- procedure used in preparing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- methods used (with citations) for chemical analyses of test material (sediment and pore water) and test water; including details concerning aliquot sampling, preparation, and storage before analysis; and
- use and description of preliminary or range-finding test.

#### 7.2.6 *Test Conditions and Procedures*

- measurements of light intensity adjacent to surface of overlying water in test chambers;
- statement concerning any aeration of overlying water in test chambers before and during the test; aeration rate and manner;
- records of any disruption of air flow to test chambers during static test, and of related DO measurements;
- description of procedure and rate for renewal of overlying water;

- appearance of each sample (or mixture thereof) and of the overlying water in test chambers; changes in appearance noted during test;

- any other chemical measurements (e.g., contaminant concentration, acid volatile sulphides, biochemical oxygen demand, chemical oxygen demand, total inorganic carbon, cation exchange capacity, redox potential, porewater hydrogen sulphide, porewater ammonia) made before and during the test on test material (including control and reference sediment) and contents of test chambers; including analyses of whole sediment, pore water, and overlying water;
- any other observations or analyses made on the test material (including samples of control or reference sediment); e.g., faunal tracks, qualitative and/or quantitative data regarding indigenous macrofauna or detritus, geochemical analyses; and
- chemical analyses of concentrations of chemical in test solutions of reference toxicant.

#### 7.2.7 *Test Results*

- results for any range-finding test(s) conducted;
- if measured, head capsule width for surviving organisms at test end, calculated for each treatment (mean  $\pm$  SD), together with the results of any statistical comparisons;



- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s);
- graphical presentation of data; and
- original bench sheets and other data sheets, signed and dated by the laboratory personnel performing the test and related analyses.

## References

---

- Acton, A.B. and G.G.E. Scudder, "The Zoogeography and Races of *Chironomus* (= *Tendipes*) *tentans* FAB", *Limnologica*, 8: 83-92 (1971).
- Ankley, G.T., D.A. Benoit, R.A. Hoke, E.N. Leonard, C.W. West, G.L. Phipps, V.R. Mattson, and L.A. Anderson, "Development and Evaluation of Test Methods for Benthic Invertebrates and Sediments: Effects of Flow Rate and Feeding on Water Quality and Exposure Conditions", *Arch. Environ. Contam. Toxicol.*, 25: 12-19 (1993).
- Ankley, G.T., D.A. Benoit, J.C. Balogh, T.B. Reynoldson, K.E. Day, and R.A. Hoke, "Evaluation of Potential Confounding Factors in Sediment Toxicity Tests with Three Freshwater Benthic Invertebrates", *Environ. Toxicol. Chem.*, 13, 627-635 (1994).
- APHA, AWWA, and WEF (American Public Health Association, American Water Works Association, and Water Environment Federation), "Toxicity", Part 8000, in: *Standard Methods for the Examination of Water and Wastewater*, 19th ed., APHA, AWWA, and WEF, Washington, DC (1995).
- Armstrong, F.A.J. and D.P. Scott, "Photochemical Dechlorination of Water Supply for Fish Tanks with Commercial Water Sterilizers", *J. Fish. Res. Board Can.*, 31: 1881-1885 (1974).
- ASTM (American Society for Testing and Materials), "Standard Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates", E1383-90, p. 1-20 in: *1991 Annual Book of ASTM Standards - Pesticides, Resource Recovery, Hazardous Substances and Oil Spill Responses, Waste Disposal, Biological Effects, Vol. 11.04*, ASTM, Philadelphia, PA (1991a).
- ASTM (American Society for Testing and Materials), "Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing", E1391-90, p. 1105-1119 in: *1991 Annual Book of ASTM Standards - Pesticides, Resource Recovery, Hazardous Substances and Oil Spill Responses, Waste Disposal, Biological Effects, Vol. 11.04*, ASTM, Philadelphia, PA (1991b).
- ASTM (American Society for Testing and Materials), "Standard Guide for Conducting Sediment Toxicity Tests with Freshwater Invertebrates", E1383-94, p. 1196-1225 in: *1994 Annual Book of ASTM Standards - Pesticides, Resource Recovery, Hazardous Substances and Oil Spill Responses, Waste Disposal, Biological Effects, Vol. 11.04*, ASTM, Philadelphia, PA (1994).
- ASTM (American Society for Testing and Materials), "Standard Test Methods

- for Measuring the Toxicity of Sediment-associated Contaminants with Freshwater Invertebrates", E1706-95, p. 1204-1285 in: *1995 Annual Book of ASTM Standards - Pesticides, Resource Recovery, Hazardous Substances and Oil Spill Responses, Waste Disposal, Biological Effects, Vol. 11.05*, ASTM, Philadelphia, PA (1995a).
- ASTM (American Society for Testing and Materials), "Standard Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing", E1706-95, p. 835-855 in: *1995 Annual Book of ASTM Standards - Pesticides, Resource Recovery, Hazardous Substances and Oil Spill Responses, Waste Disposal, Biological Effects, Vol. 11.05*, ASTM, Philadelphia, PA (1995b).
- Baudo, R., J.P. Giesy, and H. Muntau, *Sediments: Chemistry and Toxicity of In-place Pollutants*, 405 p., Lewis Publishers, Inc., Chelsea, MI (1990).
- BBA/IVA, "International Toxicity Ring-test on Sediment-dwelling *Chironomus riparius*. Protocol for the Toxicity Ring-test of Two Pesticides to the Sediment Dwelling Larvae of *Chironomus riparius*", BBA/IVA *ad hoc* Working Group on Sediment Toxicity Tests, BBA, Braunschweig, Germany (1994).
- Becker, D.S. and G.N. Bigham, "Field Validation of the Amphipod and Chironomid Freshwater Toxicity Tests", SETAC Platform Session #416, *14th Annual Meeting, Soc. Environ. Toxicol. Chem.*, Nov. 14-18, 1993, Houston, TX [Unpublished Report] (1993).
- Becker, D.S., C.D. Rose, and G.N. Bigham, "Comparison of the 10-day Freshwater Sediment Toxicity Tests Using *Hyalella azteca* and *Chironomus tentans*", *Environ. Toxicol. Chem.*, 14: 2089-2094 (1995).
- Bedard, D., "Sediment Bioassay Development: Addressing the Pros and Cons", p. 471-474 in: *Proc., Technology Transfer Conf. No. 10, Vol. 1*, November 20, 1989, Toronto, ON (1989).
- Bedard, D. and H. Ali, "Evaluation of the 10-day Sediment Toxicity Test Using the Midge (*Chironomus tentans*): the Effect of Different Water:Sediment Ratios on Biological Endpoints and Water Quality", final report, October 1996, Ontario Ministry of Environment and Energy, Etobicoke, ON (1996).
- Bedard, D., A. Hayton, and D. Persaud, "Ontario Ministry of the Environment Laboratory Sediment Biological Testing Protocol", Report No. ISBN 0-7729-9924-4, August 1992, Queen's Printer, Toronto, ON (1992).
- Benoit, D.A. and G.T. Ankley, "Methods for Conducting Life-cycle Sediment Toxicity Tests with *Chironomus tentans* Using the Newly Developed

- Sediment Testing Intermittent Renewal (STIR) System", SETAC Platform Session #420, 14th Annual Meeting, Soc. Environ. Toxicol. Chem., Nov. 14-18, 1993, Houston, TX (1993).
- Benoit, D.A., G.L. Phipps, and G.T. Ankley, "A Sediment Testing Intermittent Renewal System for the Automated Renewal of Overlying Water in Toxicity Tests with Contaminated Sediments", *Water Res.*, 27 (9): 1403-1412 (1993).
- Brouwer, H., T. Murphy, and L. McArdle, "A Sediment-contact Bioassay with *Photobacterium phosphoreum*", *Environ. Toxicol. Chem.*, 9: 1353-1358 (1990).
- Brown, A.F. and D. Pascoe, "Studies on the Acute Toxicity of Pollutants to Freshwater Macroinvertebrates - 5. The Acute Toxicity of Cadmium to Twelve Species of Predatory Macroinvertebrates", *Arch. Hydrobiol.* 114: 311-319 (1988).
- Burton, G.A. "Assessing the Toxicity of Freshwater Sediments", *Environ. Toxicol. Chem.*, 10: 1585-1627 (1991).
- Burton, G.A., Jr., ed., *Sediment Toxicity Assessment*, 457 p., Lewis Publishers, Inc., Chelsea, MI (1992).
- Burton, G.A., Jr. and C.G. Ingersoll, "Evaluating the Toxicity of Sediments", in: *The ARCS Assessment Guidance Document*, Report EPA/905-B94/002, United States Environmental Protection Agency, Chicago, IL (1994).
- Burton, G.A., Jr., T.J. Norberg-King, C.G. Ingersoll, D.A. Benoit, G.T. Ankley, P.V. Winger, J. Kubitz, J.M. Lazorchak, M.E. Smith, E. Greer, F.J. Dwyer, D.J. Call, K.E. Day, P. Kennedy, and M. Stinson, "Interlaboratory Study of Precision: *Hyalella azteca* and *Chironomus tentans* Freshwater Sediment Toxicity Assays", *Environ. Toxicol. Chem.*, 15: 1335-1343 (1996).
- Cairns, M.A., A.V. Nebeker, J.H. Gakstatter, and W.L. Griffiths, "Toxicity of Copper-spiked Sediments to Freshwater Invertebrates", *Environ. Toxicol. Chem.*, 3: 435-445 (1984).
- Call, D.J., L.T. Brooke, G.T. Ankley, D.A. Benoit, C.W. West, and R.A. Hoke, "A Short-term Method for Estimating the Toxicity of Solid Phase Sediment to *Chironomus tentans*", 61 p., Draft Report Prepared for the United States Environmental Protection Agency, Washington, DC (1993a).
- Call, D.J., K. Liber, and L.T. Brooke, "The *Chironomus tentans* Survival and Growth Bioassay: A Study of the Effect of Reduced Larval Growth Upon the Following Generation and an Application of the Bioassay in Evaluating Sediment Quality", 69 p. + app., Final Report Prepared by Lake Superior Research Institute for the United States Army Corps of

- Engineers, September 1993,  
Vicksburg, MA (1993b).
- CCREM (Canadian Council of Resource and  
Environment Ministers), "Canadian  
Water Quality Guidelines", Task  
Force on Water Quality Guidelines,  
CCREM, Environment Canada,  
Ottawa, ON (1987).
- Chapman, P.M., R.N. Dexter, S.F. Cross,  
and D.G. Mitchell, "A Field Trial of  
the Sediment Quality Triad in San  
Francisco Bay", NOAA Tech. Mem.  
NOS OMA 25, National Oceanic and  
Atmospheric Administration,  
Rockville, MD (1986).
- Chapman, P.M., R.N. Dexter, and E.R.  
Long, "Synoptic Measures of  
Sediment Contamination, Toxicity  
and Infaunal Community  
Composition (the Sediment Quality  
Triad) in San Francisco Bay",  
*Marine Ecol.*, 37: 75-96 (1987).
- Chapman, P.M., E.A. Power, R.N. Dexter,  
and H.B. Andersen, "Evaluation of  
Effects Associated with an Oil  
Platform, Using the Sediment Quality  
Triad", *Environ. Toxicol. Chem.*, 10:  
407-424 (1991).
- Coffman, W.P., "Chironomidae", Chapter  
22 in: *An Introduction to the Aquatic  
Insects of North America*, 441 p.,  
R.W. Merritt and K. W. Cummins  
(eds), Second Edition, Kendall/Hunt  
Publishing Company, Dubuque, IA  
(1984).
- Cranston, P.S. and F. Reiss, "The Larvae of  
Chironomidae (Diptera) of the  
Holarctic Region - Key to  
subfamilies", in: *Chironomidae of the  
Holarctic Region, Keys and  
Diagnoses, Part 1 - Larvae*. 457 p.  
T. Wiederholm (ed.), Entomologica  
Scandinavica Supplement No. 19  
(1983).
- Credland, P.F., "A New Method for  
Establishing a Permanent Laboratory  
Culture of *Chironomus riparius*  
Meigen (Diptera: Chironomidae)",  
*Freshwater Biol.*, 3: 45-51 (1973a).
- Credland, P.F., "The Taxonomic Status of  
*Chironomus riparius* Meigen and  
*Chironomus thummi* Kieffer  
(Diptera: Chironomidae)", *J.  
Natural History*, 7: 209-216 (1973).
- Curry, L., "A study of the Ecology and  
Taxonomy of Freshwater Midges  
(Diptera Tendipedidae) of Michigan  
with Special Reference to Their Role  
in the 'Turnover' of Radioactive  
Substances in the Hydrosol", p. 106-  
149, Report 2, Atomic Energy  
Commission and National Institutes  
of Health, Central Michigan  
University, Department of Biology,  
Mount Pleasant, MI (1962).
- Day, K.E., R.S. Kirby, and T.B. Reynoldson,  
"Sexual Dimorphism in *Chironomus  
riparius* (Meigen): Impact on  
Interpretation of Growth in Whole  
Sediment Toxicity Tests", *Environ.  
Toxicol. Chem.*, 13: 35-39 (1994).
- Day, K.E., B.J. Dutka, K.K. Kwan, N.  
Batista, T.B. Reynoldson, and J.L.  
Metcalf-Smith, "Correlations  
Between Solid-phase Microbial



- Screening Assays, Whole Sediment Toxicity Tests with Macroinvertebrates and *In-situ* Benthic Community Structure", *J. Great Lakes Res.*, 21: 192-206 (1995a).
- Day, K.E., S. Kirby, and T.B. Reynoldson, "The Effect of Manipulations of Freshwater Sediments on Responses of Benthic Invertebrates in Whole Sediment Toxicity Tests", *Environ. Toxicol. Chem.*, 14: 1333-1343 (1995b).
- de Groot, A.J. and K.H. Zschuppe, "Contribution to the Standardization of the Methods of Analysis for Heavy Metals in Sediments", *Rapp. P.-v. Reun. cons. Int. Explor. Mer.*, 181: 111-122 (1981).
- Di Toro, D.M., J.H. Mahony, D.J. Hansen, K.J. Scott, M.B. Hicks, S.M. Mayr, and M. Redmond, "Toxicity of Cadmium in Sediments: the Role of Acid Volatile Sulfides", *Environ. Toxicol. Chem.*, 9: 1487-1502 (1990).
- Di Toro, D.M., C.S. Zarba, D.J. Hansen, W.J. Berry, R.C. Swartz, C.E. Cowan, S.P. Pavlou, H.E. Allen, N.A. Thomas, and P.R. Paquin, "Technical Basis for Establishing Sediment Quality Criteria for Nonionic Organic Chemicals Using Equilibrium Partitioning", *Environ. Toxicol. Chem.*, 10: 1541-1583 (1991).
- Ditsworth, G.R., D.W. Schults, and J.K.P. Jones, "Preparation of Benthic Substrates for Sediment Toxicity Testing", *Environ. Toxicol. Chem.*, 9: 1523-1529 (1990).
- Dodge, E.E. and T.L. Theis, "Effect of Chemical Speciation on the Uptake of Copper by *Chironomus tentans*", *Environ. Sci. Technol.*, 13: 1287-1288 (1979).
- Douglas, W.S., A. McIntosh, and J.C. Clausen, "Toxicity of Sediments Containing Atrazine and Carbofuran to Larvae of the Midge *Chironomus tentans*", *Environ. Toxicol. Chem.*, 12: 847-853 (1993).
- Dwyer, F.J., C.J. Ingersoll, and N.E. Kemble, "Use of Standardized Formulated Sediment in Toxicity Tests", SETAC Platform Session #275, 14th Annual Meeting, Soc. Environ. Toxicol. Chem., Nov. 14-18, 1993, Houston, TX [Unpublished Report] (1993).
- EC (Environment Canada), "Biological Test Method: Acute Lethality Test Using *Daphnia* sp.", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/11, 57 p. (1990a).
- EC (Environment Canada), "Guidance Document on the Control of Toxicity Test Precision Using Reference Toxicants", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/12, 85 p. (1990b).
- EC (Environment Canada), "Biological Test Method: Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods", Conservation and

Protection, Ottawa, ON, Report EPS 1/RM/26, 73 p. (1992a).

EC (Environment Canada), "Biological Test Method: Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/24, 61 p. (1992b).

EC (Environment Canada), "Biological Test Method: Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/21, 73 p. (1992c).

EC (Environment Canada), "Biological Test Method: Test of Larval Growth and Survival Using Fathead Minnows", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/22, 70 p. (1992d).

EC (Environment Canada), "Biological Test Method: Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/27, 97 p. (1992e).

EC (Environment Canada), "Guidance Document on Collection and Preparation of Sediments for Physicochemical Characterization and Biological Testing", Environmental Protection Service, Ottawa, ON, Report EPS 1/RM/29, 144 p. (1994).

EC (Environment Canada), "Guidance Document on Measurement of

Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant", Environmental Protection Service, Ottawa, ON, Report EPS 1/RM/30, 56 p. (1995).

EC (Environment Canada), "Biological Test Method: Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyaella azteca*", Environmental Protection Service, Ottawa, ON, Report EPS 1/RM/33 (1997a).

EC (Environment Canada), "Biological Test Method: Test for Survival and Growth in Sediment Using Estuarine or Marine or Polychaete Worms (*Polydora cornuta* or *Boccardia proboscidea*)", Environmental Protection Service, Ottawa, ON, Report EPS x/xx/xx, in Preparation (1997b).

EC (Environment Canada), "Guidance Document on the Interpretation and Application of Data for Environmental Toxicology", Environmental Protection Service, Ottawa, ON, Report EPS 1/RM/ 34, in Preparation (1997c).

EC (Environment Canada), "Guidance Document on Statistical Methods to Determine Endpoints of Toxicity Tests", Environmental Protection Service, Ottawa, ON, Report EPS 1/RM/xx, in Preparation (1997d).

Eisenhart, C., M.W. Hastay, and W.A. Wallace, *Techniques of Statistical Analysis*, Chapter 15, McGraw-Hill Book Co., New York, NY (1947).

- Gauss, J.D., P.E. Woods, R.W. Winner, and J.H. Skillings, "Acute Toxicity of Copper to Three Life Stages of *Chironomus tentans* as Affected by Water Hardness/Alkalinity", *Environ. Pollut. (Series A)*, 37: 149-157 (1985).
- Giesey, J.P., R.L. Graney, J.L. Newsted, C.J. Rosiu, A. Benda, R.G. Kreis, Jr., and F.J. Horvath, "Comparison of Three Sediment Bioassay Methods Using Detroit River Sediments", *Environ. Toxicol. Chem.*, 7: 483-498 (1988).
- Giesey, J.P., C.J. Rosiu, and R.L. Graney, "Benthic Invertebrate Bioassays with Toxic Sediment and Pore Water", *Environ. Toxicol. Chem.*, 9: 233-248 (1990).
- Green, D.W.J., K.A. Williams, and D. Pascoe, "Studies on the Acute Toxicity of Pollutants to Freshwater Macroinvertebrates - 2. Phenol", *Arch. Hydrobiol.* 103: 75-82 (1985).
- Green, D.W.J., K.A. Williams, and D. Pascoe, "Studies on the Acute Toxicity of Pollutants to Freshwater Macroinvertebrates - 4. Lindane", *Arch. Hydrobiol.* 106: 263-273 (1986).
- Gower, A.M. and P.J. Buckland, "Water Quality and the Occurrence of *Chironomus riparius* Meigen (Diptera: Chironomidae) in a Stream Receiving Sewage Effluent" *Freshwater Biol.*, 8: 153-164 (1978).
- Hamilton, M.A., R.C. Russo, and R.V. Thurston, "Trimmed Spearman-Kärber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays", *Environ. Sci. Technol.*, 11: 714-719 (1977).
- Harrahy, E.A. and W.H. Clements, "The Effects of a Mixture of Heavy Metals in Sediment on *Chironomus tentans* (Diptera: Chironomidae)", SETAC Platform Session #505, 14th Annual Meeting, Soc. Environ. Toxicol. Chem., Nov. 14-18, 1993, Houston, TX (1993).
- Hoke, R., "Development of a Standard Protocol for *Chironomus tentans*", p. 387-392 in: *Proceedings, Tiered Testing Issues for Freshwater and Marine Sediments*, September 16-18, 1992, United States Environmental Protection Agency, Washington, DC (1992).
- Hoke, R.A., P.A. Kosian, G.T. Ankley, A.M. Cotter, F.M. Vandermeiden, G.L. Phipps, and E.J. Durhan, "Check Studies with *Hyaella azteca* and *Chironomus tentans* in Support of the Development of a Sediment Quality Criterion for Dieldrin", *Environ. Toxicol. Chem.*, 14: 435-443 (1995).
- Hubert, J.J., "PROBIT2: a Microcomputer Program for Probit Analysis", Dept. Mathematics and Statistics, Univ. Guelph, Guelph, ON [Unpublished Report] (1987).
- Ingersoll, C.G. and M.K. Nelson, "Testing Sediment Toxicity with *Hyaella azteca* (Amphipoda) and *Chironomus*

- riparius* (Diptera)", p. 93-109 in: *Aquatic Toxicology and Risk Assessment*, W.G. Landis and W.H. van der Schalle (eds). 13th Volume, ASTM STP 1096, American Society for Testing and Materials, Philadelphia, PA (1990).
- Ingersoll, C.G., F.J. Dwyer, and T.W. May, "Toxicity of Inorganic and Organic Selenium to *Daphnia magna* (Cladocera) and *Chironomus riparius* (Diptera)", *Environ. Toxicol. Chem.*, 9: 1171-1181 (1990).
- Ingersoll, C.G., G.T. Ankley, D.A. Benoit, E.L. Brunson, G.A. Burton, F.J. Dwyer, R.A. Hoke, P.F. Landrum, T.J. Norberg-King, and P.V. Winger, "Toxicity and Bioaccumulation of Sediment-associated Contaminants Using Freshwater Invertebrates: A Review of Methods and Applications", *Environ. Toxicol. Chem.*, 14: 1885-1894 (1995).
- Johannsen, O.A., "Aquatic Diptera. III. Chironomidae: Subfamilies Tanypodidae, Diamesinae and Orthocladiinae", *Memoir Cornell Univ. Agric. Exper. Station*, 205: 3-84 (1937a).
- Johannsen, O.A., "Aquatic Diptera. IV. Chironomidae: Subfamily Chironominae", *Memoir Cornell Univ. Agric. Exper. Station*, 210: 3-56 (1937b).
- Kemble, N.E., W.G. Brumbaugh, E.L. Brunson, F.J. Dwyer, C.G. Ingersoll, D.P. Monda, and D.F. Woodward, "Toxicity of Metal-contaminated Sediments from the Upper Clark Fork River, MT to Aquatic Invertebrates in Laboratory Exposures", *Environ. Toxicol. Chem.*, 13, 1985-1997 (1994).
- Kiknadze, I.I., A.I. Shilova, I.E. Kerkis, I. Kerkis, I.A. Shobanov, N.I. Zelentsov, L.P. Grebenuk, A.G. Istomina, and V.A. Prasolov, "Karyotypes and Morphology of Larvae of Tribe Chironomini", *Science*, 114 p., USSR Academy of Science, Siberian Division, Novosibirsk, USSR (1991).
- Lamberti, G.A. and J.W. Moore, "Aquatic Insects as Primary Consumers", p. 164-195, in: V.H. Resh and D.M. Rosenberg, eds., *The Ecology of Aquatic Insects*, Praeger Publishers, New York, NY (1984).
- Liber, K., D.J. Call, T.D. Dawson, F.W. Whiteman, and T.M. Dillon, "Effects of *Chironomus tentans* Larval Growth Retardation on Adult Emergence and Ovipositing Success: Implications for Interpreting Freshwater Sediment Bioassays", *Hydrobiologia*, 323: 155-167 (1996).
- Lydy, M.J., K.A. Bruner, D.M. Fry, and S.W. Fisher, "Effects of Sediment and the Route of Exposure on the Toxicity and Accumulation of Neutral Lipophilic and Moderately Water Soluble Metabolizable Compounds in the Midge, *Chironomus riparius*", p. 140-164 in: *Aquatic Toxicology and Risk Assessment*, W.G. Landis and W.H.

- van der Schalle (eds.), 13th Volume, ASTM STP 1096, American Society for Testing and Materials, Philadelphia, PA (1990).
- Lydy, M.J., J.T. Oris, P.C. Baumann, and S.W. Fisher, "Effects of Sediment Organic Carbon Content on the Elimination Rates of Neutral Lipophilic Compounds in the Midge (*Chironomus riparius*)", *Environ. Toxicol. Chem.*, 11: 347-356 (1992).
- McCahon, C.P. and D. Pascoe, "Culture Techniques for Three Freshwater Macroinvertebrate Species and Their Use in Toxicity Tests", *Chemosphere*, 17 (12): 2471-2480 (1988).
- Maki, A.W., "Modifications of Continuous Flow Test Methods for Small Aquatic Organisms", *Prog. Fish. Cult.*, 39: 172-174 (1977).
- Malloch, J.R., "A Preliminary Classification of Diptera, Exclusive of Pupipara, Based upon Larval and Pupal Characters, with Keys to Imagines in Certain Families", Part I, Bull. Illinois State Lab. Natural History, Vol XII, Urbana, IL (1917).
- Masters, J.A., M.A. Lewis, D.H. Davidson, and R.D. Bruce, "Validation of a Four-day *Ceriodaphnia* Toxicity Test and Statistical Considerations in Data Analysis", *Environ. Toxicol. Chem.*, 10: 47-55 (1991).
- Mearns, A.J., R.C. Swartz, J.M. Cummins, P.A. Dinnel, P. Plesha, and P.M. Chapman, "Interlaboratory Comparison of a Sediment Toxicity Test Using the Marine Amphipod, *Rhepoxynius abronius*", *Marine Environ. Res.*, 19: 13-37 (1986).
- Milani, D., K.E. Day, D.J. McLeay, and R.S. Kirby, "Recent Intra- and Interlaboratory Studies Related to the Development and Standardization of Environment Canada's Biological Test Methods for Measuring Sediment Toxicity Using Freshwater Amphipods (*Hyalella azteca*) or Midge Larvae (*Chironomus riparius*)", Technical report prepared by National Water Research Institute, Burlington, ON for the Method Development and Application Section, Environment Canada, Ottawa, ON (1996).
- Mosher, R.G., R.A. Kimerle, and W.J. Adams, "MIC Environmental Assessment Method for Conducting 14-day Partial Life Cycle Flow-through and Static Sediment Exposure Toxicity Tests with the Midge *Chironomus tentans*", Report No. ES-82-M-10, 12 p., August 17, 1982, Monsanto Industrial Chemicals Co., Environmental Sciences Section, St. Louis, MO (1982).
- Nebeker, A.V., M.A. Cairns, J.H. Gakstatter, K.W. Malueg, G.S. Schuytema, and D.F. Krawczyk, "Biological Methods for Determining Toxicity of Contaminated Freshwater Sediments to Invertebrates", *Environ. Toxicol. Chem.*, 3: 617-630 (1984).



- Nebeker, A.V., S.T. Onjukka, and M.A. Cairns, "Chronic Effects of Contaminated Sediment on *Daphnia magna* and *Chironomus tentans*", *Bull. Environ. Contam. Toxicol.*, 41: 574-581 (1988).
- Nelson, M.K., P.F. Landrum, G.A. Burton, S.J. Klaine, E.A. Crecelius, T.D. Byl, D.C. Gossiaux, V.N. Tsybal, L. Cleveland, C.G. Ingersoll, and G. Sasson-Brickson, "Toxicity of Contaminated Sediments in Dilution Series with Control Sediments", *Chemosphere*, 27: 1789-1812 (1994).
- Newman, M.C., Quantitative Methods in Aquatic Ecotoxicology, Lewis Publishers, Boca Raton, Florida (1995).
- Norberg-King, T.E., "Instar Development in *Chironomus tentans*", Personal Communication, United States Environmental Protection Agency, Environ. Res. Lab.-Duluth, Duluth, MN (1994).
- NWRI (National Water Research Institute), "Standard Operating Procedures: Culture and Sediment Bioassay Methods for *Chironomus riparius*, *Hexagenia* spp., *Hyaella azteca*, and *Tubifex tubifex*", K. Day, September 30, 1992 draft document,, Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON [Unpublished Report] (1992).
- Ormerod, S.J., P. Boole, C.P. McCahon, N.S. Weatherley, D. Pascoe, and R.W. Edwards, "Short-term Experimental Acidification of a Welsh Stream: Comparing the Biological Effects of Hydrogen Ions and Aluminum", *Freshwater Biol.*, 17: 341-356 (1987).
- Othoudt, R.A., J.P. Giesy, K.R. Grzyb, D.A. Verbrugge, R.A. Hoke, J.B. Drake, and D. Anderson, "Evaluation of the Effects of Storage Time on the Toxicity of Sediments", *Chemosphere*, 22: 801-807 (1991).
- Paine, M.D. and C.A. McPherson, "Phase IV Studies of 10-day Tests for Sediment Toxicity Using Marine or Estuarine Infaunal Amphipods", Unpublished Report Prepared for Environment Canada (Marine Environment Division, EP, C&P) and IGATG by EVS Consultants Ltd., North Vancouver, BC (1991).
- Pascoe, D., K.A. Williams, and D.W.J. Green, "Chronic Toxicity of Cadmium to *Chironomus riparius* Meigen - Effects Upon Larval Development and Adult Emergence", *Hydrobiologia*, 175: 109-115 (1989).
- Pascoe, D., A.F. Brown, B.M.J. Evans, and C. McKavanagh, "Effects and Fate of Cadmium During Toxicity Tests with *Chironomus riparius* - the Influence of Food and Artificial Sediment", *Arch. Environ. Contam. Toxicol.*, 19: 872-877 (1990).
- Pascoe, D., D.E. Gower, C.P. McCahon, M.J. Poulton, A.J. Whiles, and J. Wulforst, "Behavioural Responses

- to Pollutants - Application in Freshwater Bioassays", p. 246-254 in: *Bioindicators and Environmental Management*, D.W. Jeffrey and B. Madden, eds., Academic Press, New York, NY (1991).
- Phipps, G.L., V.R. Mattson, and G.T. Ankley, "The Relative Sensitivity of Three Freshwater Benthic Macroinvertebrates to Ten Contaminants", *Arch. Environ. Toxicol. Chem.*, 28: 281-286 (1995).
- Pinder, L.C.V., "The Larvae of Chironomidae (Diptera) of the Holarctic Region - Introduction", in *Chironomidae of the Holarctic Region. Keys and Diagnoses. Part 1 - Larvae*. 457 p. T. Wiederholm (ed.), Entomologica Scandinavica Supplement No. 19 (1983).
- Pittinger, C.A., D.M. Woltering, and J.A. Masters, "Bioavailability of Sediment Sorbed and Aqueous Surfactants to *Chironomus riparius* (Midge)", *Environ. Toxicol. Chem.*, 8: 1023-1033 (1989).
- Powlesland, C. and J. George, "Acute and Chronic Toxicity of Nickel to Larvae of *Chironomus riparius* (Meigen)", *Environ. Pollut. (Series A)*, 42: 47-64 (1986).
- Rasmussen, J.B., "The Life History, Distribution and Production of *Chironomus riparius* and *Glyptotendipes paripes* in a Prairie Pond", *Hydrobiologia*, 119: 65-72 (1984).
- Reynoldson, T.B., K.E. Day, C. Clarke, and D. Milani, "Effect of Indigenous Animals on Chronic End Points in Freshwater Sediment Toxicity Tests", *Environ. Toxicol. Chem.*, 13: 973-977 (1994).
- Reynoldson, T.B., R.C. Bailey, K.E. Day, and R.H. Norris, "Biological Guidelines for Freshwater Sediment Based on Benthic Assessment of Sediment (the BEAST) Using a Multivariate Approach for Predicting Biological State", *Aust. J. Ecol.*, 20: 198-219 (1995).
- RIZA, "Chironomid, Chronic Toxicity Test on Larvae in Spiked Sediment", by van de Guchte, C., E.M.M. Grootelaar, and J.L. Mass, August 1993, 20 p. Draft OECD Guideline for Testing of Chemicals, Organization for Cooperation and Economic Development. Institute for Inland Water Management and Waste Water Treatment, Riza, Netherlands (1993).
- Rocchini, R.J., M.J.R. Clark, A.J. Jordan, S. Horvath, D.J. McLeay, J.A. Servizi, A. Sholund, H.J. Singleton, R.G. Watts, and R.H. Young, "Provincial Guidelines and Laboratory Procedures for Measuring Acute Lethal Toxicity of Liquid Effluents to Fish", B.C. Ministry of Environment, Victoria, BC (1982).
- Sadler, W.O., "Biology of the Midge *Chironomus tentans* Fabricius and Methods of its Propagation",

*Memoirs Cornell Univ. Agric. Exper. Station*, 173: 1-25 (1935).

SAS (SAS Institute Inc.), "SAS procedures guide, Release 6.03, and Additional SAS/STAT procedures, Release 6/03 (SAS Technical Report P-179). SAS Institute Inc., Cary, NC (1988).

Schubauer-Berigan, M.K. and G.T. Ankley, "The Contribution of Ammonia, Metals and Nonpolar Organic Compounds to the Toxicity of Sediment Interstitial Water from an Illinois River Tributary", *Environ. Toxicol. Chem.*, 10: 925-939 (1991).

Schubauer-Berigan, M.K., P.D. Monson, C.W. West, and G.T. Ankley, "Influence of pH on the Toxicity of Ammonia to *Chironomus tentans* and *Lumbriculus variegatus*", *Environ. Toxicol. Chem.*, 14: 713-717 (1995).

Sergy, G., "Recommendations on Aquatic Biological Tests and Procedures for Environmental Protection, C & P, DOE", Environment Canada Report, Environmental Protection, Conservation and Protection, Edmonton, AB [Unpublished Report] (1987).

Sibley, P.K., D.G. Dixon, and D.R. Barton, "Comparison of Pore Water, Bulk Sediment, and Elutriate Toxicity Tests for Determining the Toxicity of Sediments Exposed to BKME to Benthic Macroinvertebrates", SETAC Platform Session #414, 14th Annual Meeting, Soc. Environ.

*Toxicol. Chem.*, Nov. 14-18, 1993, Houston, TX (1993).

Sly, P.G. and W.J. Christie, "Factors Influencing Densities and Distributions of *Pontoporeia hoyi* in Lake Ontario", *Hydrobiologia*, 235/236: 321-352 (1992).

Sokal, R.R. and F.J. Rohlf, *Biometry*, W.H. Freeman and Co., San Francisco, CA (1969).

Steel, R.G.D. and J.H. Torrie, *Principles and Procedures of Statistics*, McGraw-Hill Book Co., New York, NY (1980).

Stephan, C.E., "Methods for Calculating an LC50", p. 65-84 in: *Aquatic Toxicology and Hazard Evaluation*, F.L. Mayer and J.L. Hamelink (eds.), ASTM STP 634, American Society For Testing and Materials, Philadelphia, PA (1977).

Suedel, B.C. and J.H. Rodgers Jr., "Development of Formulated Reference Sediments for Freshwater and Estuarine Sediment Testing", *Environ. Toxicol. Chem.*, 13: 1163-1175 (1994a).

Suedel, B.C. and J.H. Rodgers Jr., "Responses of *Hyaella azteca* and *Chironomus tentans* to Particle Size Distribution and Organic Matter Content of Formulated and Natural Freshwater Sediments", *Environ. Toxicol. Chem.*, 13: 1639-1648 (1994b).

- Suedel, B.C., J.H. Rodgers Jr., and P.A. Clifford, "Bioavailability of Fluoranthene in Freshwater Sediment Toxicity Tests", *Environ. Toxicol. Chem.*, 12: 155-165 (1993a).
- Suedel, B.C., E. Deaver, and J.H. Rodgers Jr., "Reducing Uncertainty in Laboratory Sediment Toxicity Tests", SETAC Platform Session #508, 14th Annual Meeting, Soc. Environ. Toxicol. Chem., Nov. 14-18, 1993, Houston, Texas (1993b).
- Suedel, B.C., E. Deaver, and J.H. Rodgers, Jr., "Formulated Sediment as a Reference and Dilution Sediment in Definitive Toxicity Tests", *Arch. Envir. Contam. Toxicol.*, 30: 47-52 (1996).
- Suter, G.W. II, A.E. Rosen, E. Linder, and D.F. Parkhurst, "Endpoints for Responses of Fish to Chronic Toxic Exposures", *Environ. Toxicol. Chem.*, 6: 793-809 (1987).
- Taylor, E.J., S.J. Maund, and D. Pascoe, "Toxicity of Four Common Pollutants to the Freshwater Macroinvertebrates *Chironomus riparius* Meigen (Insecta: Diptera) and *Gammarus pulex* (L.) (Crustacea: Amphipoda)", *Arch. Environ. Contam. Toxicol.*, 21: 371-376 (1991a).
- Taylor, E.J., S.J. Maund, and D. Pascoe, "Evaluation of a Chronic Toxicity Test Using Growth of the Insect *Chironomus riparius* Meigen", p. 343-352 in: *Bioindicators and Environmental Management*, D.W. Jeffrey and B. Madden, eds., Academic Press, NY (1991b).
- Taylor, E.J., S.J. Blockwell, S.J. Maund, and D. Pascoe, "Effects of Lindane on the Life-cycle of a Freshwater Macroinvertebrate *Chironomus riparius* Meigen (Insecta: Diptera)", *Arch. Environ. Contam. Toxicol.*, 24: 145-150 (1993).
- Tomasovic, M., F.J. Dwyer, I.E. Greer, and C.G. Ingersoll, "Recovery of Known-age *Hyaella azteca* (Amphipoda) from Sediment Toxicity Tests", *Environ. Toxicol. Chem.*, 14: 1177-1180 (1995).
- Topping, M.S., "Ecology of Larvae of *Chironomus tentans* (Diptera: Chironomidae) in Saline Lakes in Central British Columbia", *Can. Entomol.*, 193: 328-338 (1971).
- Trussell, R.P., "The Percent Un-ionized Ammonia in Aqueous Ammonia Solutions at Different pH Levels and Temperatures", *J. Fish. Res. Board Can.*, 29: 1505-1507 (1972).
- Townes, H.K., "The Nearctic Species of Tendipedini (Diptera, Tendipedidae (= Chironomidae))", *Amer. Midland Naturalist*, 34: 1-206 (1945).
- Townsend, B.E., S.G. Lawrence, and J.F. Flannagan, "*Chironomus tentans* (Fabricius)", p. 109-126 in: *Manual for the Culture of Selected Freshwater Invertebrates*, Canad. Spec. Publ. Fish. Aquat. Sci. No. 54,

Department of Fisheries and Oceans,  
Ottawa, ON (1981).

draft, 14 p., Environ. Res. Lab.,  
USEPA, Duluth, MN (1991a).

USEPA (United States Environmental  
Protection Agency), "Methods for  
Measuring the Acute Toxicity of  
Effluents to Freshwater and Marine  
Organisms", Report EPA/600/  
4-85/013, W.H. Peltier and  
C.I. Weber, (eds.), USEPA,  
Cincinnati, OH (1985a).

USEPA (United States Environmental  
Protection Agency), "Methods for  
Measuring the Acute Toxicity of  
Effluents and Receiving Waters to  
Freshwater and Marine Organisms",  
Report EPA-600/4-90/027, USEPA,  
Cincinnati, OH (1991b).

USEPA (United States Environmental  
Protection Agency), "Short-term  
Methods for Estimating the Chronic  
Toxicity of Effluents and Receiving  
Waters to Freshwater Organisms",  
Report EPA/600/4-85/014, W.B.  
Horning and C.I. Weber (eds.),  
162p., USEPA, Cincinnati, OH  
(1985b).

USEPA (United States Environmental  
Protection Agency), "Methods for  
Measuring the Acute Toxicity of  
Effluents and Receiving Waters to  
Freshwater and Marine Organisms",  
Report EPA-600/4-90/027F, Office  
of Research and Development,  
Environmental Monitoring Systems  
Laboratory, USEPA, Cincinnati,  
Ohio (1993).

USEPA (United States Environmental  
Protection Agency), "Ambient Water  
Quality Criteria for Ammonia -  
1984", Report EPA 440/5-85-001,  
USEPA, Washington, DC (1985c).

USEPA (United States Environmental  
Protection Agency), "Methods for  
Measuring the Toxicity and  
Bioaccumulation of Sediment-  
associated Contaminants with  
Freshwater Invertebrates", Report  
EPA 600/R-94/024, 133 p., USEPA,  
Duluth, MN (1994a).

USEPA (United States Environmental  
Protection Agency), "Short-term  
Methods for Estimating the Chronic  
Toxicity of Effluents and Receiving  
Waters to Freshwater Organisms",  
2nd ed., Report EPA/600/4-89/001,  
248 p., USEPA, Cincinnati, OH  
(1989).

USEPA (United States Environmental  
Protection Agency), "Short-term  
Methods for Estimating the Chronic  
Toxicity of Effluents and Receiving  
Waters to Freshwater Organisms",  
3rd ed., Report EPA/600/4-91-002,  
341 p., USEPA, Cincinnati, OH  
(1994b).

USEPA (United States Environmental  
Protection Agency), "Standard  
Operating Procedure for the Culture  
of *Chironomus tentans*", J. Denny  
and K. Mead, (eds.), Oct. 29, 1991

USEPA/USACE (United States  
Environmental Protection  
Agency/United States Army Corps of



- Engineers), "Evaluation of Dredged Material Proposed for Discharge in Waters of the United States - Testing Manual (Draft)", Report EPA-823-B-94-002, prepared by USEPA/USACE, Washington, DC (1994).
- Usinger, R.L., "*Aquatic Insects of California, With Keys to North American Genera and California Species*", 508 p., Univ. Calif. Press, Berkeley and Los Angeles, CA (1963).
- Wentzel, R., A. McIntosh, and G. Atchison, "Sublethal Effects of Heavy Metal Contaminated Sediment on Midge Larvae (*Chironomus tentans*)", *Hydrobiologia*, 56 (2): 153-156 (1977a).
- Wentzel, R., A. McIntosh, W.P. McCafferty, G. Atchinson, and V. Anderson, "Avoidance Response of Midge Larvae (*Chironomus tentans*) When Exposed to Sediments Containing Heavy Metals", *Hydrobiologia*, 55: 171-175 (1977b).
- Wentzel, R., A. McIntosh, and P.C. McCafferty, "Emergence of the Midge *Chironomus tentans* When Exposed to Heavy Metal Contaminated Sediments", *Hydrobiologia*, 57: 195-196 (1978).
- WEST, Inc., and D.D. Gulley, "Toxstat® 3.5", Western EcoSystems Technology, Inc., Cheyenne, WY [Computer software and instruction manual. WEST, Inc., 203 Central Ave., Cheyenne, WY, 82001, USA] (1996).
- West, C.W., V.R. Mattson, E.N. Leonard, G.L. Phipps, and G.T. Ankley, "Comparison of the Relative Sensitivity of Three Benthic Invertebrates to Copper-contaminated Sediments from the Keweenaw Waterway", *Hydrobiologia*, 262: 57-63 (1993).
- Whiteman, F.W., G.T. Ankley, M.D. Kahl, D.M. Rau, and M.D. Balcer, "Evaluation of Interstitial Water as a Route of Exposure for Ammonia in Sediment Tests with Benthic Macroinvertebrates", *Environ. Toxicol. Chem.*, 15: 794-801 (1996).
- Williams, D.A., "A Test for Differences Between Treatment Means when Several Dose Levels are Compared with a Zero Dose Control", *Biometrics*, 27: 103-118 (1971).
- Williams, D.A. "The Comparison of Several Dose Levels with a Zero Dose Control", *Biometrics*, 28: 519-532 (1972).
- Williams, K.A., D.W. Green, And D. Pascoe, "Studies on the Acute Toxicity of Pollutants to Freshwater Macroinvertebrates - 1. Cadmium", *Arch. Hydrobiol.* 102: 461-471 (1985).
- Williams, K.A., D.W. Green, And D. Pascoe, "Studies on the Acute Toxicity of Pollutants to Freshwater Macroinvertebrates - 3. Ammonia",

*Arch. Hydrobiol.* 106: 61-70  
(1986a).

Williams, K.A., D.W.J. Green, D. Pascoe,  
and D.E. Gower, "The Acute  
Toxicity of Cadmium to Different  
Larval Stages of *Chironomus*  
*riparius* (Diptera: Chironomidae) and  
its Ecological Significance for  
Pollution Regulation", *Oecologia*  
(Berlin), 70: 362-366 (1986b).

Williams, K.A., D.W.J. Green, D. Pascoe,  
and D.E. Gower, "Effect of

Cadmium on Oviposition and Egg  
Viability in *Chironomus riparius*  
(Diptera: Chironomidae)", *Bull.*  
*Environ. Contam. Toxicol.*, 38: 86-  
90 (1987).

Zumwalt, D.C., F.J. Dwyer, I.E. Greer, and  
C.G. Ingersoll, "A Water-renewal  
System that Accurately Delivers  
Small Volumes of Water to Exposure  
Chambers", *Environ. Toxicol. Chem.*,  
13: 1311-1314 (1994).

*Appendix A*

---

**Members of the Inter-Governmental Aquatic Toxicity Group  
(as of July 1997)*****Federal, Environment Canada***

N. Bermingham  
Centre St. Laurent  
Montreal, PQ

C. Blaise  
Centre St. Laurent  
Montreal, PQ

S. Blenkinsopp  
Environmental Technology Centre  
Edmonton, AB

C. Boutin  
National Wildlife Research Centre  
Hull, PQ

A. Chevrier  
Marine Environment Division  
Hull, PQ

A. Cook  
Newfoundland District  
St. John's, NF

K. Day  
National Water Research Institute  
Burlington, ON

K. Doe  
Environmental Conservation Branch  
Moncton, NB

G. Elliott  
Ecotoxicology Laboratory  
Edmonton, AB

P. Jackman  
Environmental Conservation Branch  
Moncton, NB

R. Kent  
Evaluation and Interpretation Branch  
Hull, PQ

N. Kruper  
Ecotoxicology Laboratory  
Edmonton, AB

R. Legault  
Centre St. Laurent  
Montreal, PQ

D. MacGregor  
Environmental Technology Centre  
Gloucester, ON

D. Moul  
Pacific Environmental Science Centre  
North Vancouver, BC

W.R. Parker  
Atlantic Region  
Dartmouth, NS

L. Porebski  
Marine Environment Division  
Hull, PQ

D. Rodrigue  
Environmental Technology Centre  
Gloucester, ON

R. Scroggins  
Environmental Technology Centre  
Gloucester, ON

A. Steenkamer  
Environmental Technology Centre  
Gloucester, ON

G. van Aggelen  
Pacific Environmental Science Centre  
North Vancouver, BC

R. Watts  
Pacific Environmental Science Centre  
North Vancouver, BC

P. Wells  
Atlantic Region  
Dartmouth, NS

W. Windle  
Commercial Chemicals and Evaluation  
Branch  
Hull, PQ

S. Yee  
Pacific Environmental Science Centre  
North Vancouver, BC

***Federal, Fisheries and Oceans Canada***  
D. Cyr  
Institut Maurice la Montagne  
Mont Joli, PQ

***Federal, Natural Resources Canada***  
B. McAfee  
CANMET  
Ottawa, ON

***Federal, Atomic Energy Control Board***  
P. Thompson  
Radiation and Protection Division  
Ottawa, ON

***Provincial***  
S. Abernethy  
Ministry of Environment and Energy  
Etobicoke, ON

C. Bastien  
Min. de l'env. et de la faune  
Ste-Foy, PQ

D. Bedard  
Ministry of Environment and Energy  
Etobicoke, ON

J. Lee  
Ministry of Environment and Energy  
Etobicoke, ON

M. Mueller  
Ministry of Environment and Energy  
Etobicoke, ON

C. Neville  
Ministry of Environment and Energy  
Etobicoke, ON

D. Poirier  
Ministry of Environment and Energy  
Etobicoke, ON

G. Westlake  
Ministry of Environment and Energy  
Etobicoke, ON

*Appendix B*

---

**Environment Canada, Environmental Protection Service,  
Regional and Headquarters Offices****Headquarters**

351 St. Joseph Boulevard  
Place Vincent Massey  
Hull, Quebec  
K1A 0H3

**Ontario Region**

2<sup>nd</sup> Floor  
4905 Dufferin Street  
Downsview, Ontario  
M3H 5T4

**Atlantic Region**

15th Floor, Queen Square  
45 Alderney Drive  
Dartmouth, Nova Scotia  
B2Y 2N6

**Western and Northern Region**

Room 210, Twin Atria No. 2  
4999 - 98th Avenue  
Edmonton, Alberta  
T6B 2X3

**Quebec Region**

14<sup>th</sup> Floor  
105 McGill Street  
Montreal, Quebec  
H2Y 2E7

**Pacific and Yukon Region<sup>52</sup>**

224 Esplanade Street  
North Vancouver, British Columbia  
V7M 3H7

---

<sup>52</sup> A BASIC computer program for calculating LC50 is available from the Aquatic Toxicology Section, Pacific Environmental Science Centre, 2645 Dollarton Highway, North Vancouver, BC, V7H 1V2, by providing a formatted computer diskette.



## Appendix C

---

### Procedural Variations for Culturing *Chironomus* sp., as Described in Canadian, United States, and European Methodology Documents

Source documents are listed chronologically, by originating agency. They can be accessed as:

**USEPA 1984** represents Nebeker *et al.* (1984). This publication gives the culturing and test methods used at that time by the USEPA Environmental Research Laboratory at Corvallis, Oregon for determining the toxicity of contaminated freshwater sediments to a number of freshwater invertebrates including *C. tentans*.

**UWCC 1988** represents McCahon and Pascoe (1988). This publication gives the culture techniques used in 1988 at the University of Wales College of Cardiff (UK) for culturing a number of freshwater invertebrates including *C. riparius* in preparation for acute and chronic toxicity tests.

**USFWS 1990** represents Ingersoll and Nelson (1990). This publication gives the culturing and sediment toxicity test procedures for *C. riparius* and *Hyaella azteca* then in use by the National Fisheries Contaminant Research Center, U.S. Fish and Wildlife Service, Columbia, MO. Some details were taken from Ingersoll *et al.* (1990).

**ASTM 1991** is the standard guide published by the American Society for Testing and Materials for conducting sediment toxicity tests with freshwater invertebrates including *C. tentans* and *C. riparius*. This guideline document was published again in subsequent years including 1994. See "ASTM 1991a" and "ASTM 1994" in list of references.

**USEPA 1991a** is the draft (October 29, 1991) standard operating procedure for culturing *C. tentans*, then used by the USEPA Environmental Research Laboratory at Duluth, MN. See "USEPA 1991a" in list of references.

**NWRI 1992** is the (unpublished) standard operating procedures used in 1992 for culturing and testing *C. riparius* and other freshwater invertebrates, by K. Day of the National Water Research Institute (NWRI), Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON. See "NWRI 1992" in list of references.

**OME 1992** represents Bedard *et al.* (1992). This publication gives the laboratory procedures then in use by the Water Resources Branch, Ontario Ministry of Environment, for culturing freshwater invertebrates including *C. tentans*, and for conducting static sediment toxicity tests with each species. These procedures were designed to enable direct comparison of test results for concurrent, single-species sediment assays for toxicity and bioaccumulation using *C. tentans* larvae, mayfly (*Hexagenia limbata*) nymphs, and fathead minnows.

USEPA 1992 is a synthesis of information found in Hoke (1992) and Appendix A of USEPA (1994a), and represents the response of 11 laboratories to a questionnaire sent by USEPA Office of Science and Technology, and Office of Research and Development, Washington, DC, regarding procedures used for culturing *C. tentans* or *C. riparius* in preparation for sediment toxicity tests.

RIZA 1993 is the draft procedure for culturing *C. riparius* and for conducting sediment toxicity tests, developed by the Institute for Inland Water Management and Waste Water Treatment (RIZA; Lelystad, the Netherlands) for consideration by the Organization for Economic Cooperation and Development as an OECD guideline for testing of chemicals. See "RIZA 1993" in list of references.

USEPA 1993 represents Call *et al.* (1993a). This unpublished report gives the draft method for culturing *C. tentans* and conducting sediment toxicity tests with this species, that was prepared for and by the USEPA.

BBA/IVA 1994 is the procedure used for an international comparative assay with pesticides and *C. riparius*. See "BBA/IVA 1994" in list of references.

Sheffield 1994 is the unpublished standard operating procedures used by C. Naylor and co-workers of the Dept. of Animal and Plant Sciences, University of Sheffield, Sheffield, U.K.

USEPA 1994a is the published methods for culturing *C. tentans* and other freshwater invertebrates, and for conducting sediment toxicity tests, developed by USEPA (principal authors, C.G. Ingersoll, G.T. Ankley, G.A. Burton, F.J. Dwyer, T.J. Norberg-King, and P.V. Winger). See "USEPA 1994a" in list of references.

## 1. Source of Brood Stock for Culture

| Document <sup>a</sup> | Species                                   | Initial Source                                                                                       |
|-----------------------|-------------------------------------------|------------------------------------------------------------------------------------------------------|
| USEPA 1984            | <i>C. tentans</i>                         | NI <sup>b</sup>                                                                                      |
| UWCC 1988             | <i>C. riparius</i>                        | NI                                                                                                   |
| USFWS 1990            | <i>C. riparius</i>                        | NI                                                                                                   |
| ASTM 1991             | <i>C. tentans</i> ,<br><i>C. riparius</i> | natural freshwater source, another laboratory, or<br>a commercial supplier                           |
| USEPA 1991a           | <i>C. tentans</i>                         | egg cases from other laboratories                                                                    |
| NWRI 1992             | <i>C. riparius</i>                        | laboratory culture (C. Ingersoll)                                                                    |
| OME 1992              | <i>C. tentans</i>                         | cultures at University of Toronto (N. Collins, R. Baker) and<br>Michigan State University (J. Giesy) |
| USEPA 1992            | <i>C. tentans</i> ,<br><i>C. riparius</i> | NI (various, depending on the laboratory)                                                            |
| RIZA 1993             | <i>C. riparius</i>                        | egg cases from other laboratories, or a wild population                                              |
| USEPA 1993            | <i>C. tentans</i>                         | natural freshwater source, or another laboratory                                                     |
| BBA/IVA 1994          | <i>C. riparius</i>                        | NI                                                                                                   |
| Sheffield 1994        | <i>C. riparius</i>                        | another laboratory in UK                                                                             |
| USEPA 1994a           | <i>C. tentans</i>                         | another laboratory, a commercial supplier, or a natural<br>population from a clean area <sup>c</sup> |

<sup>a</sup> See preceding page for correct citation.<sup>b</sup> NI = Not indicated.<sup>c</sup> Obtaining organisms from wild populations should be avoided, unless the ability of the wild population to cross-breed with existing laboratory populations has been demonstrated.

## 2. Culture Vessel, Water Volume, and Organisms per Vessel at Start

| Document       | Species                                   | Vessel Type <sup>a</sup>                 | Water Volume (L) | Organisms per Vessel at Start  |
|----------------|-------------------------------------------|------------------------------------------|------------------|--------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | 10-L glass aquarium                      | NI <sup>b</sup>  | 2 egg masses                   |
| UWCC 1988      | <i>C. riparius</i>                        | 4-L plastic<br>(17 × 17 × 17 cm)         | 1.3              | 10 hatched egg ropes           |
| USFWS 1990     | <i>C. riparius</i>                        | 30 × 30 × 30 cm plastic                  | 3                | ≥ 3 egg cases                  |
| ASTM 1991      | <i>C. tentans</i>                         | glass aquarium, 3 to 19 L                | NI <sup>c</sup>  | NI                             |
|                | <i>C. riparius</i>                        | 30 × 30 × 30 cm plastic                  | 3                | ≥ 3 egg cases                  |
| USEPA 1991a    | <i>C. tentans</i>                         | 36 × 21 × 26 cm tank                     | 6 to 8           | 3 hatched egg cases            |
| NWRI 1992      | <i>C. riparius</i>                        | 20-L glass aquarium                      | NI <sup>d</sup>  | 3 hatched egg ropes            |
| OME 1992       | <i>C. tentans</i>                         | 21-L aquarium<br>(40 × 20 × 25 cm)       | 8 <sup>d</sup>   | 250 2nd-instar larvae          |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | 1- to 80-L aquarium                      | 1 to 30          | 50 to 800 <sup>e</sup>         |
| RIZA 1993      | <i>C. riparius</i>                        | plastic aquarium                         | 3 to 5           | a few (e.g., 5) egg cases      |
| USEPA 1993     | <i>C. tentans</i>                         | 19-L glass aquarium<br>(36 × 21 × 26 cm) | 7.5              | 2 to 3 hatched egg masses      |
| BBA/IVA 1994   | <i>C. riparius</i>                        | NI <sup>f</sup>                          | NI               | 2 to 4 egg masses              |
| Sheffield 1994 | <i>C. riparius</i>                        | 23 × 12 × 8 cm plastic <sup>g</sup>      | 1.0              | 100 larvae, <24-h old          |
| USEPA 1994a    | <i>C. tentans</i>                         | 19-L glass aquarium<br>(36 × 21 × 26 cm) | 7.5              | 150 to 200 larvae <sup>h</sup> |

<sup>a</sup> All culture vessels are covered with fine-mesh screening to prevent the escape of emerged adults.

<sup>b</sup> NI = Not indicated.

<sup>c</sup> Water added to a depth of 45 cm.

<sup>d</sup> Water added to a depth of ~10 cm.

<sup>e</sup> As egg cases or <24-h old larvae.

<sup>f</sup> Water added to a depth of several cm.

<sup>g</sup> A series of small boxes are contained within a large glass-topped cage to prevent escape of adults.

<sup>h</sup> Five egg masses will provide adequate numbers of larvae to start a new culture.

### 3. Water Source, Hardness, and Method of Replacement During Culturing

| Document       | Species                                   | Water Source                                                    | Water Hardness (mg/L) | Method of Replacement                                                  |
|----------------|-------------------------------------------|-----------------------------------------------------------------|-----------------------|------------------------------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | NI <sup>a</sup>                                                 | NI                    | FT <sup>b</sup> , or IR <sup>c</sup> (portion, once weekly)            |
| UWCC 1988      | <i>C. riparius</i>                        | dechl. tap <sup>d</sup>                                         | NI                    | NI                                                                     |
| USFWS 1990     | <i>C. riparius</i>                        | well                                                            | 283                   | IR (~3 volume renewals/day)                                            |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | well, surface, dechl. tap <sup>e</sup> , or recon. <sup>f</sup> | optional              | IR (25 to 30%/week), or FT                                             |
| USEPA 1991a    | <i>C. tentans</i>                         | filtered surface                                                | NI                    | timed FT (1 volume renewal/day), or IR (50% every 2 to 3 days)         |
| NWRI 1992      | <i>C. riparius</i>                        | filtered dechl. tap                                             | 119 to 137            | IR (replace evaporation losses)                                        |
| OME 1992       | <i>C. tentans</i>                         | dechl. tap                                                      | 144                   | IR (20% every 2 weeks)                                                 |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | dechl. tap(3), well(5), lake (1), recon. (1)                    | soft to very hard     | IR (8) or FT (2)                                                       |
| RIZA 1993      | <i>C. riparius</i>                        | recon.                                                          | 210                   | IR (replace evaporation losses)                                        |
| USEPA 1993     | <i>C. tentans</i>                         | various                                                         | optional              | FT ( $\geq 1$ volume renewal/day) or IR (1 volume renewal/4 to 7 days) |
| BBA/IVA 1994   | <i>C. riparius</i>                        | well, dechl., recon. <sup>g</sup>                               | NI                    | IR (replace evaporation losses)                                        |
| Sheffield 1994 | <i>C. riparius</i>                        | recon. <sup>g</sup>                                             | NI                    | IR (replace evaporation losses)                                        |
| USEPA 1994a    | <i>C. tentans</i>                         | well, surface, dechl. tap <sup>e</sup> , recon. <sup>h</sup>    | optional              | IR or FT <sup>i</sup>                                                  |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> FT = Flow-through.

<sup>c</sup> IR = Intermittent renewal.

<sup>d</sup> Dechlorinated municipal tap water.

<sup>e</sup> Dechlorinated water should only be used as a last resort, since dechlorination is often incomplete.

<sup>f</sup> Reconstituted water.

<sup>g</sup> A recipe is provided for preparing suitable reconstituted water ("Elendt M7", Sheffield 1994; "Elendt M4 or M7", BBA/IVA 1994).

<sup>h</sup> A recipe is provided for preparing suitable reconstituted water with hardness 90 to 100 mg/L.

<sup>i</sup> Renewal of culture water, with at least one volume addition/d, is recommended. As a minimum, the overlying water volume should be changed at least weekly by siphoning.



## 4. Temperature, Aeration, and Lighting During Culturing

| Document       | Species                                   | Water Temp.<br>(°C) | Aeration Conditions    | Lighting                              |
|----------------|-------------------------------------------|---------------------|------------------------|---------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | 20                  | yes if IR <sup>a</sup> | 16L:8D <sup>b</sup>                   |
| UWCC 1988      | <i>C. riparius</i>                        | 18 to 20            | yes                    | natural daylight                      |
| USFWS 1990     | <i>C. riparius</i>                        | 20 ± 2              | gentle (~2 bubbles/s)  | 16L:8D, 269 to 538 lux                |
| ASTM 1991      | <i>C. tentans</i>                         | 20 to 23            | gentle, if IR          | 16L:8D, 538 lux                       |
|                | <i>C. riparius</i>                        | 20 to 22            | gentle, if IR          | 16L:8D, 538 lux                       |
| USEPA 1991a    | <i>C. tentans</i>                         | 23                  | maintain DO >5 mg/L    | 16L:8D, fluor. <sup>c</sup> , 330 lux |
| NWRI 1992      | <i>C. riparius</i>                        | 23 ± 1              | gentle                 | NI <sup>d</sup>                       |
| OME 1992       | <i>C. tentans</i>                         | 20 ± 2              | continuous             | 16L:8D, fluor.                        |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | 19 to 25            | yes (moderate)         | 16L:8D, 500 to 1300 lux               |
| RIZA 1993      | <i>C. riparius</i>                        | 20 ± 1              | yes (moderate)         | 16L:8D <sup>e</sup>                   |
| USEPA 1993     | <i>C. tentans</i>                         | 23                  | likely yes if IR       | 16L:8D                                |
| BBA/TVA 1994   | <i>C. riparius</i>                        | 20                  | yes                    | 16L:8D, 1000 lux                      |
| Sheffield 1994 | <i>C. riparius</i>                        | 20 ± 2              | gentle                 | 14L:10D, fluor. <sup>c</sup>          |
| USEPA 1994a    | <i>C. tentans</i>                         | 23                  | yes if static or IR    | 16L:8D, 500 to 1000 lux               |

<sup>a</sup> IR = Intermittent renewal.<sup>b</sup> Daily photoperiod of 16 hours light and 8 hours dark.<sup>c</sup> Overhead fluorescent tubes.<sup>d</sup> NI = Not indicated.<sup>e</sup> Intensity is not critical.

## 5. Substrate for Larval Midges During Culturing

| Document       | Species                                   | Description of Substrate Used                                                            | Size/Quantity of Substrate                    |
|----------------|-------------------------------------------|------------------------------------------------------------------------------------------|-----------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | Cerophyll™ (provides substrate and food)                                                 | 100 to 200 mg/aquarium <sup>a</sup>           |
| UWCC 1988      | <i>C. riparius</i>                        | homogenized cellulose mulch <sup>b</sup>                                                 | 3-cm depth/aquarium                           |
| USFWS 1990     | <i>C. riparius</i>                        | sand with mean grain size ~0.5 mm                                                        | thin layer                                    |
| ASTM 1991      | <i>C. tentans</i><br><i>C. riparius</i>   | homogenized pulp from brown paper towels<br>ground cereal leaves (as substrate and food) | 3-cm depth/aquarium<br>200 to 300 mg/aquarium |
| USEPA 1991a    | <i>C. tentans</i>                         | homogenized pulp from white paper towels                                                 | 500 mL pulp/aquarium                          |
| NWRI 1992      | <i>C. riparius</i>                        | silica sand (thoroughly washed)                                                          | 1.5 to 2 cm depth/aquarium                    |
| OME 1992       | <i>C. tentans</i>                         | fine-grade silica sand (thoroughly washed)                                               | 1.6 L/aquarium                                |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | unbleached paper towels, 7 labs; bleached paper towels, 1 lab; sand, 2 labs              | NI <sup>c</sup>                               |
| RIZA 1993      | <i>C. riparius</i>                        | sterile sediment from unpolluted location <sup>d</sup>                                   | ~150 g (damp)/aquarium                        |
| USEPA 1993     | <i>C. tentans</i>                         | bleached or unbleached paper towels, or sand                                             | ~150 mL pulp/aquarium                         |
| BBA/TVA        | <i>C. riparius</i>                        | silica sand                                                                              | ~0.5 to 1 cm depth/aquarium                   |
| Sheffield 1994 | <i>C. riparius</i>                        | sand                                                                                     | ~2 cm depth/aquarium                          |
| USEPA 1994a    | <i>C. tentans</i>                         | silica sand, or pulp from paper towels                                                   | ~150 mL pulp/aquarium                         |

<sup>a</sup> Initially, 100 mg is added when eggs hatch, and three days later. Thereafter, 200 mg is added 2×/week.

<sup>b</sup> One sheet of Whatman No. 1 filter paper is homogenized in 350 mL water, then 1 L water is added.

<sup>c</sup> NI = Not indicated.

<sup>d</sup> Microfauna must be removed by filtering through 500 µm sieve. Sediment may be irradiated.

## 6. Feeding During Culturing

| Document       | Species                                   | Description of Food Used                                                                                                         | Quantity <sup>a</sup>                       | Feeding Frequency         |
|----------------|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------|---------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | Cerophyll <sup>TM</sup>                                                                                                          | 100 to 200 mg <sup>b</sup>                  | 2×/week                   |
| UWCC 1988      | <i>C. riparius</i>                        | finely ground Tetramin <sup>TM</sup> fish food                                                                                   | 300 mg                                      | daily                     |
| USFWS 1990     | <i>C. riparius</i>                        | mixture of Cerophyll <sup>TM</sup> , <i>S. capricornutum</i> <i>ad libitum</i> , and Hartz <sup>TM</sup> Dog Treats <sup>c</sup> | 300 mg <sup>c</sup>                         | daily                     |
| ASTM 1991      | <i>C. tentans</i><br><i>C. riparius</i>   | Cerophyll <sup>TM</sup> and/or fish food flakes as per USFWS 1990 <sup>c</sup>                                                   | various <sup>d</sup><br>300 mg <sup>c</sup> | 2×/day<br>daily           |
| USEPA 1991a    | <i>C. tentans</i>                         | ground Tetrafin <sup>TM</sup> goldfish food                                                                                      | 5 mL slurry                                 | daily                     |
| NWRI 1992      | <i>C. riparius</i>                        | ground Nutrafin <sup>TM</sup> fish food flakes                                                                                   | 200 to 500 mg <sup>e</sup>                  | daily                     |
| OME 1992       | <i>C. tentans</i>                         | Cerophyll <sup>TM</sup> and Tetra Conditioning Food <sup>TM</sup>                                                                | 5 to 20 mL slurry <sup>f</sup>              | varied <sup>f</sup>       |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | various (Tetramin <sup>TM</sup> , Nutrafin <sup>TM</sup> , YCT and algae, alfalfa and Tetrafin <sup>TM</sup> )                   | various                                     | 3 to 7×/week              |
| RIZA 1993      | <i>C. riparius</i>                        | various (e.g., Tetramin <sup>TM</sup> or Aquariaan <sup>TM</sup> )                                                               | various <sup>g</sup>                        | 1 to 3×/week <sup>g</sup> |
| USEPA 1993     | <i>C. tentans</i>                         | ground Tetrafin <sup>TM</sup> goldfish food                                                                                      | 300 mg                                      | daily                     |
| BBA/TVA 1994   | <i>C. riparius</i>                        | ground Tetramin <sup>TM</sup> fish food                                                                                          | 250 mg                                      | daily                     |
| Sheffield 1994 | <i>C. riparius</i>                        | ground Tetramin <sup>TM</sup> fish food                                                                                          | 100 mg                                      | 3×/week                   |
| USEPA 1994a    | <i>C. tentans</i>                         | ground Tetrafin <sup>TM</sup> goldfish food                                                                                      | 300 mg                                      | daily                     |

<sup>a</sup> Amount of food added to each culture vessel.

<sup>b</sup> When eggs hatch (0 d) and again at 3 d, 100 mg Cerophyll<sup>TM</sup> plus an optional 10 mg of powdered Tetra<sup>TM</sup> fish food is placed in the aquarium; 200 mg Cerophyll<sup>TM</sup> (plus 10% Tetra<sup>TM</sup>) is added twice weekly thereafter.

<sup>c</sup> To establish a culture, Cerophyll<sup>TM</sup> (300 mg) is added once, together with the freshwater alga *Selenastrum capricornutum*, *ad libitum*. Thereafter, 300 mg of a suspension of Hartz Dog Treats<sup>TM</sup> was added daily.

<sup>d</sup> Amount depends on the number and size of larvae. New cultures should receive ≤0.5 mL of a suspension of 1 g dry fish food flakes mixed in 10 mL of culture water and refrigerated.

<sup>e</sup> Larvae 0 to 5 days old are fed 0.2 to 0.3 g/aquarium; older larvae are fed 0.4 - 0.5 g/aquarium.

<sup>f</sup> A fine slurry of Cerophyll<sup>TM</sup> powder and Tetra<sup>TM</sup> is prepared (3:2 ratio). Larvae are initially fed 10 to 20 mL of this slurry, and then 5 mL every other day. The ration and feeding interval is adjusted as larvae grow.

<sup>g</sup> Feed 1st instars ~50 mg one time only, 2nd instars ~100 mg 3×/week, and 3rd and 4th instars ~500 mg 3×/week.

## Appendix D

---

### Procedural Variations for Sediment Toxicity Tests Using *Chironomus* sp., as Described in Canadian, United States, and European Methodology Documents

Source documents are listed chronologically by originating agency. They can be accessed as:

**USEPA 1984** represents Nebeker *et al.* (1984). This publication gives the culturing and test methods used at that time by the USEPA Environmental Research Laboratory at Corvallis, Oregon for determining the toxicity of contaminated freshwater sediments to a number of freshwater invertebrates including *C. tentans*.

**USFWS 1990** represents Ingersoll and Nelson (1990). This publication gives the culturing and sediment toxicity test procedures for *C. riparius* and *Hyalella azteca* then in use by the National Fisheries Contaminant Research Center, United States Fish and Wildlife Service, Columbia, MO. Some details were taken from Ingersoll *et al.* (1990).

**ASTM 1991** is the standard guide published by the American Society for Testing and Materials for conducting sediment toxicity tests with freshwater invertebrates including *C. tentans* and *C. riparius*. This guideline document was published again in subsequent years including 1994. See "ASTM 1991a" and "ASTM 1994" in list of references.

**NWRI 1992** is the (unpublished) standard operating procedures used in 1992 for culturing and testing *C. riparius* and other freshwater invertebrates, by K. Day of the National Water Research Institute (NWRI), Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON. See "NWRI 1992" in list of references.

**OME 1992** represents Bedard *et al.* (1992). This publication gives the laboratory procedures then in use by the Water Resources Branch, Ontario Ministry of Environment, for culturing freshwater invertebrates including *C. tentans*, and for conducting static sediment toxicity tests with each species. These procedures were designed to enable direct comparison of test results for concurrent, single-species sediment assays for toxicity and bioaccumulation using *C. tentans* larvae, mayfly (*Hexagenia limbata*) nymphs, and fathead minnows.

**USEPA 1992** is a synthesis of information found in Hoke (1992) and Appendix A of USEPA (1994a), and represents the response of eight laboratories to a questionnaire sent by USEPA Office of Science and Technology, and Office of Research and Development, Washington, DC, regarding procedures used for conducting sediment toxicity tests for effects on survival and growth of *C. tentans* or *C. riparius*.

**RIZA 1993** is the draft procedure for culturing *C. riparius* and for conducting sediment toxicity tests, developed by the Institute for Inland Water Management and Waste Water Treatment (RIZA; Lelystad, the Netherlands) for consideration by the Organization for Economic Cooperation and

Development as an OECD guideline for testing of chemicals. See "RIZA 1993" in list of references.

USEPA 1993 represents Call *et al.* (1993a). This unpublished report gives the draft method for culturing *C. tentans* and conducting sediment toxicity tests with this species, that was prepared for and by the USEPA.

BBA/IVA 1994 is the procedure used for an international comparative assay with pesticides and *C. riparius*. See "BBA/IVA 1994" in list of references.

Sheffield 1994 is the unpublished standard operating procedures used by C. Naylor and co-workers of the Department of Animal and Plant Sciences, University of Sheffield, Sheffield, UK.

USEPA 1994a is the published methods for culturing *C. tentans* and other freshwater invertebrates, and for conducting sediment toxicity tests, developed by USEPA (principal authors, C.G. Ingersoll, G.T. Ankley, G.A. Burton, F.J. Dwyer, T.J. Norberg-King, and P.V. Winger). See "USEPA 1994a" in list of references.



## 1. Test Type and Duration; Age and Instar of Test Organisms at Start

| Document <sup>a</sup> | Species                                   | Test Type            | Test Duration (days)  | Age at Start                | Larval Instar           |
|-----------------------|-------------------------------------------|----------------------|-----------------------|-----------------------------|-------------------------|
| USEPA 1984            | <i>C. tentans</i>                         | S <sup>b</sup>       | 15                    | 10-d old                    | second                  |
| USFWS 1990            | <i>C. riparius</i>                        | FT <sup>c</sup>      | 13                    | <24-h old                   | first                   |
| ASTM 1991             | <i>C. tentans</i>                         | S or FT              | 10 to 14              | 10- to 15-d old             | second                  |
|                       | <i>C. riparius</i>                        | S or FT              | 10 to 14              | ≤24-h or 3-d old            | first                   |
| NWRI 1992             | <i>C. riparius</i>                        | S                    | 10                    | <48-h old                   | first                   |
| OME 1992              | <i>C. tentans</i>                         | S                    | 10                    | 10- to 12-d old             | second                  |
| USEPA 1992            | <i>C. tentans</i> ,<br><i>C. riparius</i> | S or FT <sup>d</sup> | 10 to 14 <sup>e</sup> | 0- to 16-d old <sup>f</sup> | second <sup>f</sup>     |
| RIZA 1993             | <i>C. riparius</i>                        | IR <sup>g</sup>      | 28                    | 0-h old <sup>h</sup>        | pre-larval <sup>h</sup> |
| USEPA 1993            | <i>C. tentans</i>                         | IR<br>or FT          | 10                    | 11- to 13-d old             | second                  |
| BBA/TVA 1994          | <i>C. riparius</i>                        | S                    | ~25                   | 1- to 3-d old               | first                   |
| Sheffield 1994        | <i>C. riparius</i>                        | S or FT              | 10                    | <96-h old                   | second                  |
| USEPA 1994a           | <i>C. tentans</i>                         | IR<br>or FT          | 10                    | NI <sup>i,j</sup>           | third <sup>j</sup>      |

<sup>a</sup> See preceding page for correct citation.

<sup>b</sup> S = Static.

<sup>c</sup> FT = Flow-through.

<sup>d</sup> Six of eight laboratories surveyed routinely conducted static tests.

<sup>e</sup> Five of eight laboratories surveyed used a test duration of 10 days.

<sup>f</sup> Seven of eight laboratories surveyed used second instar larvae that were 10- to 14-days old.

<sup>g</sup> IR = Intermittent renewal.

<sup>h</sup> To start a test, parts of egg packets are distributed randomly among treatments.

<sup>i</sup> NI = Not indicated.

<sup>j</sup> At least 50% of the larvae must be in the third instar at the start of the test.

## 2. Test Vessels and Materials

| Document       | Species                                   | Vessel                                       | Cover                       | Amount of Sediment                           | Amount of Water                              |
|----------------|-------------------------------------------|----------------------------------------------|-----------------------------|----------------------------------------------|----------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | 20-L aquarium, or 4-L jar                    | NI <sup>a</sup>             | 2 to 3 cm layer                              | 15-cm layer                                  |
| USFWS 1990     | <i>C. riparius</i>                        | 1-L glass beaker                             | none <sup>b</sup>           | 200 mL                                       | 800 mL                                       |
| ASTM 1991      | <i>C. tentans</i><br><i>C. riparius</i>   | various <sup>c</sup><br>various <sup>d</sup> | NI<br>watch glass if static | various <sup>c</sup><br>various <sup>d</sup> | various <sup>c</sup><br>various <sup>d</sup> |
| NWRI 1992      | <i>C. riparius</i>                        | 250-mL glass beaker                          | petri dish                  | 50 mL                                        | 200 mL                                       |
| OME 1992       | <i>C. tentans</i>                         | 1.8-L glass jar                              | plastic or glass lids       | 325 mL                                       | 1300 mL                                      |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | various <sup>e</sup>                         | NI                          | 10 to 200 mL                                 | 40 to 1800 mL                                |
| RIZA 1993      | <i>C. riparius</i>                        | 100-mL vessel                                | NI                          | ~10 mL                                       | ~40 mL                                       |
| USEPA 1993     | <i>C. tentans</i>                         | 300-mL beaker <sup>f</sup>                   | 60-mesh SS screen           | 100 mL                                       | ~150 mL                                      |
| BBA/TVA 1994   | <i>C. riparius</i>                        | 2- to 3-L beaker                             | yes                         | 2-cm layer                                   | 15- to 20-cm layer                           |
| Sheffield 1994 | <i>C. riparius</i>                        | 60-mL glass jar                              | mesh                        | ~10 mL <sup>h</sup>                          | 50 mL                                        |
| USEPA 1994a    | <i>C. tentans</i>                         | 300-mL beaker <sup>g</sup>                   | NI                          | 100 mL                                       | 175 mL                                       |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> A cover glass was used on Day 0, during which time the overlying water was aerated and the test was static (no replacement of overlying water). Water flow was started on Day 1, at which time the cover was removed and the flow of air discontinued.

<sup>c</sup> A number of suitable test vessels are described. These range from 50-mL plastic centrifuge tubes containing 7.5 g sediment and 47 mL water for testing individual midges, to 20-L aquaria containing a 2 to 3 cm layer of sediment and a 15-cm layer of overlying water for testing groups of 100 larvae.

<sup>d</sup> Suitable test vessels include 1-L glass beakers, each containing 50 larvae, 200 mL sediment and 800 mL overlying water; and 13-L glass aquaria, each containing 130 larvae, 2 L sediment and 11 L overlying water.

<sup>e</sup> A survey of eight laboratories showed tests vessels ranging from 50-mL plastic tubes to 2-L glass jars, sediment volumes from 10 to 200 mL, and volumes of overlying water from 40 to 1800 mL. Six of the eight laboratories maintained a sediment:water ratio of 1:4, the lowest ratio was 1:1.

<sup>f</sup> High-form, lipless beaker, with two opposing, screened holes (1.5-cm dia. centered 7.7 cm above the bottom).

<sup>g</sup> High-form, lipless beaker.

<sup>h</sup> Exact amount added, based on dry weight.

### 3. Volume of Test Vessel, Number of Organisms per Vessel, and Number of Replicates per Treatment

| Document       | Species                                   | Vessel Volume (mL)       | No. per Vessel       | No. of Replicates    |
|----------------|-------------------------------------------|--------------------------|----------------------|----------------------|
| USEPA 1984     | <i>C. tentans</i>                         | 20 000                   | 100                  | ≥2                   |
| USFWS 1990     | <i>C. riparius</i>                        | 1 000                    | 50                   | 4                    |
| ASTM 1991      | <i>C. tentans</i>                         | 20 000                   | 100                  | NI <sup>a</sup>      |
| ASTM 1991      | <i>C. tentans</i>                         | 3 000                    | 25                   | NI                   |
| ASTM 1991      | <i>C. tentans</i>                         | 2 000                    | 20                   | NI                   |
| ASTM 1991      | <i>C. tentans</i>                         | 50                       | 1                    | ≤15                  |
| ASTM 1991      | <i>C. riparius</i>                        | 13 000                   | 130                  | NI                   |
| ASTM 1991      | <i>C. riparius</i>                        | 1 000                    | 50                   | NI                   |
| NWRI 1992      | <i>C. riparius</i>                        | 250                      | 15                   | 5                    |
| OME 1992       | <i>C. tentans</i>                         | 1 800                    | 15                   | ≥3                   |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | 50 to 2 000 <sup>b</sup> | 1 to 80 <sup>c</sup> | 2 to 15 <sup>d</sup> |
| RIZA 1993      | <i>C. riparius</i>                        | 100                      | 25 <sup>e</sup>      | 4                    |
| USEPA 1993     | <i>C. tentans</i>                         | 300                      | 10                   | 5                    |
| BBA/TVA 1994   | <i>C. riparius</i>                        | 2 000 to 3 000           | 25                   | 3                    |
| Sheffield 1994 | <i>C. riparius</i>                        | 60                       | 1                    | ≤10                  |
| USEPA 1994a    | <i>C. tentans</i>                         | 300                      | 10                   | 8 <sup>f</sup>       |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> Survey results of eight laboratories showed the following test-vessel volumes: 50 mL (2 labs), 250 mL (2 labs), 300 mL (1 lab), 1000 mL (2 labs), and 2000 mL (1 lab).

<sup>c</sup> 10 to 15 organisms per vessel was most common.

<sup>d</sup> 3 to 4 replicates per treatment was most common.

<sup>e</sup> At the first renewal (i.e., after 7 d), 25 larvae are transferred to each replicate vessel.

<sup>f</sup> Depends on test objective. Eight replicates are recommended for routine testing.

#### 4. Water Source, Hardness, and Method of Replacement During Test

| Document       | Species                                   | Water Source                                            | Water Hardness       | Method of Replacement           |
|----------------|-------------------------------------------|---------------------------------------------------------|----------------------|---------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | NI <sup>a</sup>                                         | NI                   | static with top up <sup>b</sup> |
| USFWS 1990     | <i>C. riparius</i>                        | recon. <sup>c</sup>                                     | 134 mg/L             | static or FT <sup>d,e</sup>     |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | well, surface, dechl.<br>tap, or recon.                 | optional             | static or FT                    |
| NWRI 1992      | <i>C. riparius</i>                        | dechl. tap <sup>h</sup>                                 | NI                   | static with top up <sup>b</sup> |
| OME 1992       | <i>C. tentans</i>                         | dechl. tap <sup>f,g</sup>                               | 144 mg/L             | static with top up <sup>b</sup> |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | natural or recon. <sup>i</sup>                          | soft to<br>very hard | static or renewal <sup>j</sup>  |
| RIZA 1993      | <i>C. riparius</i>                        | recon.                                                  | 210 mg/L             | IR, 1×/week                     |
| USEPA 1993     | <i>C. tentans</i>                         | culture water <sup>k</sup>                              | optional             | IR, 4×/d <sup>l</sup>           |
| BBA/TVA 1994   | <i>C. riparius</i>                        | well, dechl., recon.                                    | optional             | static with top up <sup>b</sup> |
| Sheffield 1994 | <i>C. riparius</i>                        | recon. ("M7")                                           | as for culture       | static or FT                    |
| USEPA 1994a    | <i>C. tentans</i>                         | culture, well, site,<br>surface, or recon. <sup>m</sup> | optional             | IR or FT, 2×/d <sup>n</sup>     |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> Distilled water was added as needed to keep water level constant.

<sup>c</sup> Reconstituted. Well water with hardness 283 mg/L was used for culturing the test organisms.

<sup>d</sup> FT = Flow-through.

<sup>e</sup> 3.8 volume additions per beaker, per day.

<sup>f</sup> Dechlorinated municipal tap water.

<sup>g</sup> Suitable sources can include uncontaminated well water.

<sup>h</sup> Same source and hardness as used for culturing organisms.

<sup>i</sup> Well water, five labs; dechlor. water, three labs; lake water, one lab; recon. water, one lab

<sup>j</sup> Of the eight laboratories surveyed, six performed static tests. Replacement rates for the renewal test varied from four times per day to three times per week.

<sup>k</sup> Alternatively, uncontaminated water with characteristics similar to culture water may be used.

<sup>l</sup> IR = Intermittent renewal. Automated exchange system set to provide ~4 volume additions per day.

<sup>m</sup> A recipe was provided for preparing suitable reconstituted water with hardness 90 to 100 mg CaCO<sub>3</sub>/L.

<sup>n</sup> Each test chamber should receive two volume additions per day of overlying water, using an intermittent renewal (manual or automated) or continuous flow system for replacements.

## 5. Temperature, Aeration, and Lighting During Test

| Document       | Species                                   | Water Temp. (°C)      | Aeration Conditions                | Lighting                                            |
|----------------|-------------------------------------------|-----------------------|------------------------------------|-----------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | 20                    | gentle aeration of overlying water | 16L:8D <sup>g</sup>                                 |
| USFWS 1990     | <i>C. riparius</i>                        | 20 ± 2                | gentle or none <sup>b</sup>        | 16L:8D, 269 to 538 lux                              |
| ASTM 1991      | <i>C. tentans</i>                         | 20 to 23 <sup>c</sup> | gentle if static                   | 16L:8D, 538 lux                                     |
|                | <i>C. riparius</i>                        | 20 to 22 <sup>c</sup> | gentle if static                   | 16L:8D, 538 lux                                     |
| NWRI 1992      | <i>C. riparius</i>                        | 23 ± 1                | gentle aeration                    | 16L:8D                                              |
| OME 1992       | <i>C. tentans</i>                         | 20 ± 2                | aerate <sup>d</sup>                | 16L:8D, fluorescent                                 |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | 20 to 25 <sup>e</sup> | moderate or none <sup>f</sup>      | 16L:8D, ~500 to 1300 lux                            |
| RIZA 1993      | <i>C. riparius</i>                        | 20 ± 1 <sup>g</sup>   | aerate 3×/week <sup>h</sup>        | 16L:8D                                              |
| USEPA 1993     | <i>C. tentans</i>                         | 22 to 23 ± 1          | none                               | 16L:8D, 538 to 1076 lux                             |
| BBA/TVA 1994   | <i>C. riparius</i>                        | 20 ± 2                | gentle aeration                    | 16L:8d, ~1000 lux                                   |
| Sheffield 1994 | <i>C. riparius</i>                        | 20 ± 2                | gentle if static                   | 16L:8D                                              |
| USEPA 1994a    | <i>C. tentans</i>                         | 23 ± 1 <sup>c</sup>   | normally, none <sup>i</sup>        | 16L:8D, ~500 to 1000 lux, wide-spectrum fluorescent |

<sup>a</sup> Daily photoperiod of 16 hours light and 8 hours dark.

<sup>b</sup> For static tests, the overlying water was aerated gently (~2 bubbles/s). For flow-through tests, gentle aeration was provided on Day 0, but was discontinued when the flow was started on Day 1.

<sup>c</sup> The daily mean temperature must be within ±1 °C of the desired temperature. Instantaneous temperature must be within ±3 °C of the desired temperature.

<sup>d</sup> Provide continuous aeration of overlying water at a rate that ensures oxygen saturation. Bubble air through Pasteur pipettes that extend 1 cm below the water surface.

<sup>e</sup> Test temperatures were 20 °C (two labs), 22 °C (two labs), 23 °C (two labs), or 25 °C (two labs).

<sup>f</sup> Of the eight laboratories surveyed, six aerated the overlying water (static tests).

<sup>g</sup> Water temperature must not differ more than ±0.5 °C between test vessels at any time during the test.

<sup>h</sup> Aerate the overlying water in all test vessels three times a week, for about 10 minutes.

<sup>i</sup> Aerate if dissolved oxygen in overlying water drops below 40% of saturation.



## 6. Feeding During Test

| Document       | Species                                   | Description of Food Used                                                 | Quantity per Vessel                          | Feeding Frequency                                 |
|----------------|-------------------------------------------|--------------------------------------------------------------------------|----------------------------------------------|---------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | Cerophyll™ and crushed Tetramin™ flakes                                  | 600 mg C, 100 mg T                           | Days 0 + 8                                        |
| USFWS 1990     | <i>C. riparius</i>                        | suspension of Cerophyll™, Hartz Dog Treats™, and <i>S. capricornutum</i> | 75 mg C + 30 mg HDT <sup>a</sup>             | daily <sup>a</sup>                                |
| ASTM 1991      | <i>C. tentans</i><br><i>C. riparius</i>   | Cerophyll™ and/or fish food flakes<br>various <sup>c</sup>               | various <sup>b</sup><br>various <sup>c</sup> | 1 to 7×/week <sup>b</sup><br>various <sup>c</sup> |
| NWRI 1992      | <i>C. riparius</i>                        | Nutrafin™ fish food flakes                                               | 8 mg <sup>d</sup>                            | 2×/week                                           |
| OME 1992       | <i>C. tentans</i>                         | Cerophyll™ and Tetra™ Conditioning Food                                  | 30 mg <sup>e</sup>                           | daily <sup>e</sup>                                |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | various <sup>f</sup>                                                     | various <sup>f</sup>                         | various <sup>f</sup>                              |
| RIZA 1993      | <i>C. riparius</i>                        | various (e.g., Tetramin™, Trouvit™, or Aquariaan™)                       | various <sup>g</sup>                         | various <sup>g</sup>                              |
| USEPA 1993     | <i>C. tentans</i>                         | Tetrafin™ goldfish food                                                  | 4 mg <sup>h</sup>                            | daily                                             |
| BBA/IVA 1994   | <i>C. riparius</i>                        | Tetramin™ fish food flakes                                               | ~58 mg <sup>i</sup>                          | ≥ 3×/week                                         |
| Sheffield 1994 | <i>C. riparius</i>                        | Tetramin™ fish food flakes                                               | 10 mg                                        | Day 0                                             |
| USEPA 1994a    | <i>C. tentans</i>                         | Tetrafin™ goldfish food                                                  | ™6 mg <sup>j</sup>                           | daily                                             |

<sup>a</sup> Plus  $6 \times 10^7$  algal cells, Day 0. Thereafter, daily additions (in addition to algae) of 15 mg Cerophyll™ from Days 1 to 6, and 30 mg of Dog Treats™ from Days 1 to 12.

<sup>b</sup> Depends on investigator, larval size, and number of organisms per test vessel. For instance, groups of 25 larvae in 3-L vessels were each fed 50 mg fish food flakes daily, whereas individual larvae in centrifuge tubes were fed a 0.1 mL suspension of 0.06 g/mL goldfish.

<sup>c</sup> Food types used include one or more of Cerophyll™, fish food flakes, commercial dog treats, and algae. Quantities fed depend on larval size and number of organisms per test vessel. Feeding frequency is daily or every other day.

<sup>d</sup> Added as a slurry of ground Nutrafin™, prepared by adding 1 g flakes to 100 mL distilled water and pulverizing. A volume of ~604  $\mu$ L is equivalent to 8 mg.

<sup>e</sup> 0.9 g Cerophyll™ and 0.6 g crushed Tetra™ are blended into a slurry using 100 mL water. Each test vessel is given 2 mL of this slurry, daily. Feeding is reduced or interrupted if fouling or food buildup occurs.

<sup>f</sup> Depending on laboratory, larvae are fed daily or two to three times per week using a choice of Tetramin™, Nutrafin™, YCT and algae, or alfalfa and Tetrafin™.

<sup>g</sup> First instars are fed once on Day 4; other instars are fed three times per week. Using a 2% suspension, first instars are fed 50  $\mu$ L, second instars 100  $\mu$ L, third instars 150  $\mu$ L, and fourth instars 200  $\mu$ L, each feeding occasion.

<sup>h</sup> Larvae in each test vessel are fed 1.0 mL of a 4 mg/mL suspension of Tetrafin™, daily.

<sup>i</sup> Feeding rate equivalent to 1 mg/larva/day (i.e., total of 175 mg/week), fed at least three times per week.

<sup>j</sup> Larvae in each test vessel are fed 1.5 mL of a 4 mg/mL suspension of Tetrafin™, daily.

## 7. Monitoring Quality of Overlying Water During Test

| Document       | Species                                   | Variables Monitored <sup>a</sup>                                 | Frequency                                                                                                            |
|----------------|-------------------------------------------|------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | DO pH alk hard                                                   | NI <sup>b</sup>                                                                                                      |
| USFWS 1990     | <i>C. riparius</i>                        | DO pH alk hard cond                                              | ≤ 10 days, each treatment <sup>c</sup>                                                                               |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | DO<br>pH alk hard cond<br>temp                                   | beginning and end, as a minimum <sup>d,e</sup><br>beginning and end, as a minimum <sup>d</sup><br>daily <sup>d</sup> |
| NWRI 1992      | <i>C. riparius</i>                        | DO pH cond temp                                                  | Days 0, 5, and 10                                                                                                    |
| OME 1992       | <i>C. tentans</i>                         | DO pH cond<br>temp<br>optional: alk hard ammonia                 | Days 0, 5, and 10<br>daily<br>NI                                                                                     |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | NI                                                               | NI                                                                                                                   |
| RIZA 1993      | <i>C. riparius</i>                        | DO pH nitrite nitrate ammonia<br>temp<br>hard cond               | beginning, each renewal, end<br>≥ 3×/week<br>beginning                                                               |
| USEPA 1993     | <i>C. tentans</i>                         | DO temp<br>pH alk hard cond<br>ammonia                           | daily<br>beginning and end <sup>d</sup><br>near beginning and end <sup>d</sup>                                       |
| BBA/TVA 1994   | <i>C. riparius</i>                        | DO pH temp                                                       | weekly                                                                                                               |
| Sheffield 1994 | <i>C. riparius</i>                        | DO pH temp                                                       | Days 0, 5, and 10                                                                                                    |
| USEPA 1994a    | <i>C. tentans</i>                         | DO <sup>g</sup><br>pH <sup>g</sup> alk hard cond ammonia<br>temp | daily<br>beginning and end <sup>h</sup> daily <sup>d,f</sup>                                                         |

<sup>a</sup> DO = dissolved oxygen; pH = hydrogen ion concentration; alk = total alkalinity; hard = total hardness;

<sup>b</sup> cond = specific conductivity; temp = temperature.

<sup>c</sup> NI = Not indicated.

<sup>d</sup> 50-mL volume of overlying water removed for measurements. In static exposure, this was replaced with fresh, temperature-adjusted overlying water.

<sup>e</sup> Measured in at least one test vessel representing each treatment.

<sup>f</sup> DO to be measured if any interruption of air (static test) or water (flow-through test) and whenever behaviour of animals indicates DO too low (e.g., if midges emerged from sediment).

<sup>g</sup> Daily mean temperature must be within ±1 °C of desired temperature; instantaneous temperature must be within ±3 °C of desired temperature.

<sup>h</sup> Can be measured directly, using a probe.

<sup>i</sup> Overlying water should be sampled just before water renewal from about 1 to 2 cm above sediment surface, using a pipette. Values should not vary by more than 50% during a test.

## 8. Storage and Characterization of Sediment Used in Test

| Document       | Species                                   | Storage Conditions                              | Characteristics Measured <sup>a</sup>                                                              |
|----------------|-------------------------------------------|-------------------------------------------------|----------------------------------------------------------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | 4°C for ≤2 weeks                                | optional: TOC SSC TA MET SOC                                                                       |
| USFWS 1990     | <i>C. riparius</i>                        | 4°C in Teflon™ bags by 24 h; use within 2 weeks | TOC IC W SSC MET PCB PAH                                                                           |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | 4 ± 2°C for ≤2 weeks <sup>b</sup>               | at least pH TOC W SSC; might include BOD COD IC TVS AVS Eh OG OS TA M PAH PW                       |
| NWRI 1992      | <i>C. riparius</i>                        | sealed plastic buckets, 4°C; use within 6 weeks | TOC IC SSC MET TKN                                                                                 |
| OME 1992       | <i>C. tentans</i>                         | 4°C for ≤4 weeks                                | TVS TOC W SSC P TKN MET OCP; might include CB PAH CDF CP                                           |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | NI <sup>c</sup>                                 | NI                                                                                                 |
| RIZA 1993      | <i>C. riparius</i>                        | 4°C for ≤3 months                               | TOC W SSC MET PCB PAH P TKN OG                                                                     |
| USEPA 1993     | <i>C. tentans</i>                         | NI                                              | NI                                                                                                 |
| BBA/TVA 1994   | <i>C. riparius</i>                        | not required (formulated)                       | NI                                                                                                 |
| Sheffield 1994 | <i>C. riparius</i>                        | 4°C for ≤2 weeks <sup>d</sup>                   | TOC IC W SSC                                                                                       |
| USEPA 1994a    | <i>C. tentans</i>                         | 4°C for ≤2 weeks <sup>e</sup>                   | at least porewater pH + TA, and TOC W SSC; might include BOD COD CEC IC TVS AVS Eh OG SOC M PAH PW |

<sup>a</sup> TOC = total organic carbon; IC = inorganic carbon; W = % water; SSC = % sand, silt, and clay; pH = hydrogen-ion concentration; BOD = biochemical oxygen demand; COD = chemical oxygen demand; CEC = cation exchange capacity; TVS = total volatile solids; SG = specific gravity (g/mL); AVS = acid volatile sulfides; Eh = oxidation reduction potential; OG = oil and grease; OS = organosilicones; TA = total ammonia; MET = metals (e.g., As, Cd, Cr, Cu, Hg, Ni, Pb, Zn); PCB = total polychlorinated biphenyls; PAH = polycyclic aromatic hydrocarbons; SOC = synthetic organic compounds; PW = porewater analyses; P = total phosphorus; TKN = total Kjeldahl nitrogen; OCP = organochlorine pesticides; CB = chlorinated benzenes; CDF = chlorinated dioxins and furans; CP = chlorinated phenols.

<sup>b</sup> If stored longer than two weeks, sediment should be retested to confirm that toxicity has not changed.

<sup>c</sup> NI = Not indicated.

<sup>d</sup> Most current work uses formulated sediment, in which case sediment is not stored before use.

<sup>e</sup> Start test as soon as possible following sample collection. If toxicity test is started after two weeks of collection, it is desirable to conduct additional characterizations of sediment to evaluate possible effects of storage.

## 9. Manipulation of Sediment Before Use in Test

| Document       | Species                                   | Sediment Manipulation                                                                                                                                                                                                                                                                                                                                        |
|----------------|-------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | mix thoroughly; screen through one or more standard sieve screens to remove large particles and endemic animals                                                                                                                                                                                                                                              |
| USFWS 1990     | <i>C. riparius</i>                        | mixed in storage container; aliquot smoothed with Teflon™ spoon in test beaker; overlying water poured gently along side of beaker; allowed to settle overnight before adding midge larvae                                                                                                                                                                   |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | mix thoroughly; may be press-sieved to remove large particles and indigenous organisms; may be diluted and mixed in a 1:1 ratio with overlying water to facilitate sieving; smooth as layer in test vessel; pour overlying water gently along side of beaker; allow to settle overnight before adding midge larvae                                           |
| NWRI 1992      | <i>C. riparius</i>                        | wet-sieve through 250 µm mesh using portion of overlying water for test; discard residue retained and leave sieved sediment and overlying water undisturbed overnight; decant overlying water for use in test; add sediment to replicate beakers and then overlying water; allow to settle 24 h and aerate minimum of 1 h before adding midge larvae         |
| OME 1992       | <i>C. tentans</i>                         | decant excess water from sediment; press sediment through a 2 mm sieve to remove large debris and any large organisms; homogenize sieved sediment with a stainless steel spoon; distribute as even (2 cm) layer in beaker by tapping side of beaker; gently add overlying water; allow to settle overnight; aerate minimum of 1 h before adding midge larvae |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | NI <sup>b</sup>                                                                                                                                                                                                                                                                                                                                              |
| RIZA 1993      | <i>C. riparius</i>                        | filter through 500 µm sieve; irradiate if necessary; thoroughly homogenize; mix with dilution water in 1:4 ratio; spike with toxicant; shake mechanically for ≥20 h; allow to settle for 1 to 3 days before adding test organisms                                                                                                                            |
| USEPA 1993     | <i>C. tentans</i>                         | sieve through coarse-mesh (e.g., 5 mm) screen; thoroughly homogenize; add to beaker; allow to settle for 24 h before adding test organisms                                                                                                                                                                                                                   |
| BBA/IVA 1994   | <i>C. riparius</i>                        | artificial sediment mixed, added to beaker, carefully flooded with control/dilution water and left ≥1 week before organisms added                                                                                                                                                                                                                            |
| Sheffield 1994 | <i>C. riparius</i>                        | mix, including any separated water; samples should not be sieved; remove large organisms and large debris using forceps; allow to settle for 24 h before adding test organisms                                                                                                                                                                               |
| USEPA 1994a    | <i>C. tentans</i>                         | mix, including any separated water; samples should not be sieved <sup>c</sup> ; remove large organisms and large debris using forceps                                                                                                                                                                                                                        |

<sup>a</sup> In some situations, the sieved sediment is frozen for 24 h and thawed, in order to kill all eggs of tubificids.

<sup>b</sup> NI = Not indicated.

<sup>c</sup> If sediment must be sieved, samples should be taken before and after sieving (e.g., pore-water metals) to document the influence of sieving on sediment chemistry.

## 10. Terminating Test and Biological Endpoints

| Document       | Species                                   | Terminating Test                                                                                                                                         | Biological Endpoints                                    |
|----------------|-------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | sieve sediment to collect larvae; kill with warm water, blot dry, and weigh/measure                                                                      | percent survival<br>mean wet weight<br>mean body length |
| USFWS 1990     | <i>C. riparius</i>                        | wet-sieved sediment in 5 stages; preserved animals in sugar-formalin for subsequent measurement                                                          | percent survival<br>head capsule width                  |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | sieve sediment using 500 $\mu\text{m}$ mesh screen; rinse; count, and weigh <sup>b</sup>                                                                 | mean percent survival<br>mean dry weight                |
| NWRI 1992      | <i>C. riparius</i>                        | sieve contents of beaker through 500 $\mu\text{m}$ mesh screen; count and weigh surviving organisms                                                      | mean percent survival<br>mean dry weight                |
| OME 1992       | <i>C. tentans</i>                         | sieve contents of test vessel; rinse; count and blot dry surviving organisms; weigh individuals to nearest 0.01 mg                                       | mean percent survival<br>wet weight                     |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | NI <sup>c</sup>                                                                                                                                          | survival, weight <sup>f</sup>                           |
| RIZA 1993      | <i>C. riparius</i>                        | sieve sediment in test vessel through 250 $\mu\text{m}$ screen; count; dry survivors; weigh                                                              | survival, dry weight<br>of fourth instars               |
| USEPA 1993     | <i>C. tentans</i>                         | sieve sediment in test vessel through 0.59 $\mu\text{m}$ screen; count; oven dry for $\geq 2$ h at 100°C until constant weight; weigh survivors as group | mean percent survival<br>mean dry weight                |
| BBA/IVA 1994   | <i>C. riparius</i>                        | 10 days after last adult emergence or 90% control emergence                                                                                              | percent adult emergence<br>time to emergence            |
| Sheffield 1994 | <i>C. riparius</i>                        | transfer sediment to petri dish; remove larvae with forceps                                                                                              | survival, length,<br>wet/dry weight                     |
| USEPA 1994a    | <i>C. tentans</i>                         | sieve sediment through 710 $\mu\text{m}$ mesh screen or using multiple sieves; count survivors; measure dry weight as group <sup>g</sup>                 | mean percent survival<br>mean dry weight <sup>g</sup>   |

<sup>a</sup> Animals not recovered are presumed to have died and decomposed.

<sup>b</sup> Dry weight (dried at 60°C to constant weight) is preferable to length. Growth can also be estimated by measuring head capsule width.

<sup>c</sup> Surviving animals from each beaker are dried for 24 h at 60°C, then weighed as a group.

<sup>d</sup> Alternatively, dry weight can be obtained after drying to constant weight. Length can also be used as an indicator of growth.

<sup>e</sup> NI = Not indicated.

<sup>f</sup> Of the 8 laboratories surveyed, 7 used dry weight at test end as the indicator of growth, and 1 used length.

<sup>g</sup> Pool survivors and dry at 60 to 90°C to constant weight, bring to room temperature in dessicator and weigh to nearest 0.01 mg. Measurement of length is optional.



## 11. Use of Control/Reference Sediment and Requirement for Valid Test

| Document       | Species                                   | Control/Reference Sediment                                                                         | Requirement for Valid Test                                                      |
|----------------|-------------------------------------------|----------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
| USEPA 1984     | <i>C. tentans</i>                         | collect and use uncontaminated control sediment <sup>a</sup>                                       | NI <sup>b</sup>                                                                 |
| USFWS 1990     | <i>C. riparius</i>                        | fine-grained control sediment used                                                                 | NI                                                                              |
| ASTM 1991      | <i>C. tentans</i> ,<br><i>C. riparius</i> | every test requires a clean (negative) control sediment or a clean reference sediment <sup>c</sup> | mean control survival $\geq 70\%$ <sup>d</sup>                                  |
| NWRI 1992      | <i>C. riparius</i>                        | clean control and reference sediments used                                                         | mean control survival $\geq 80\%$                                               |
| OME 1992       | <i>C. tentans</i>                         | clean control and reference sediments used                                                         | mean control survival $\geq 75\%$                                               |
| USEPA 1992     | <i>C. tentans</i> ,<br><i>C. riparius</i> | clean control sediment used                                                                        | control survival 70 to 80% <sup>e</sup>                                         |
| RIZA 1993      | <i>C. riparius</i>                        | clean reference sediment used                                                                      | mean control survival $\geq 90\%$<br>mean dry weight $\geq 0.3$ mg <sup>f</sup> |
| USEPA 1993     | <i>C. tentans</i>                         | negative control and reference sediments used                                                      | mean control survival $\geq 70\%$ <sup>g</sup>                                  |
| BBA/IVA 1994   | <i>C. riparius</i>                        | artificial sediment used                                                                           | mean emergence of control adults, $\geq 70\%$                                   |
| Sheffield 1994 | <i>C. riparius</i>                        | clean reference or formulated sediment used                                                        | mean control survival $\geq 80\%$                                               |
| USEPA 1994a    | <i>C. tentans</i>                         | negative control and reference sediments used                                                      | mean control survival $\geq 70\%$<br>mean dry weight $\geq 0.6$ mg <sup>h</sup> |

<sup>a</sup> Particle size and organic content should be similar to the sample(s) of contaminated sediment.

<sup>b</sup> NI = Not indicated.

<sup>c</sup> A reference sediment should be collected from the field in a clean area, and represent the test sediment in sediment characteristics (e.g., particle size, total organic carbon, pH).

<sup>d</sup> The test is unacceptable if the average survival of organisms in any test vessel containing negative control sediment is less than 70%.

<sup>e</sup> Of the 8 laboratories surveyed, 4 required a control survival of  $\geq 70\%$ , and the rest  $\geq 75$  to 80%.

<sup>f</sup> Average size of fourth instar *C. riparius* in the control sediment must be at least 0.3 mg at test end.

<sup>g</sup> Based on mean survival rate for all control replicates combined.

<sup>h</sup> Average size of *C. tentans* in the control sediment must be at least 0.6 mg at the end of the test.

## Appendix E

---

# Procedural Variations for Reference Toxicity Tests using *Chironomus* sp., as Described in Canadian, United States, and European Methodology Documents

Source documents are listed chronologically by originating agency. They can be accessed as:

**USEPA 1984** represents Nebeker *et al.* (1984). This publication gives the culturing and test methods used at that time by the USEPA Environmental Research Laboratory at Corvallis, OR for determining the toxicity of contaminated freshwater sediments to a number of freshwater invertebrates including *C. tentans*.

**USFWS 1990** represents Ingersoll and Nelson (1990). This publication gives the culturing and sediment-toxicity test procedures for *C. riparius* and *Hyaella azteca* then in use by the National Fisheries Contaminant Research Center, United States Fish and Wildlife Service, Columbia, MO.

**ASTM 1991** is the standard guide published by the American Society for Testing and Materials for conducting sediment toxicity tests with freshwater invertebrates including *C. tentans* and *C. riparius*. This guideline document was published again in subsequent years including 1994. See "ASTM 1991a" and "ASTM 1994" in list of references.

**NWRI 1992** is the (unpublished) standard operating procedures used in 1992 for culturing and testing *C. riparius* and other freshwater invertebrates, by K. Day of the National Water Research Institute (NWRI), Rivers Research Branch, Canada Centre for Inland Waters, Environment Canada, Burlington, ON. See "NWRI 1992" in list of references.

**OME 1992** represents Bedard *et al.* (1992). This publication gives the laboratory procedures then in use by the Water Resources Branch, Ontario Ministry of Environment, for culturing freshwater invertebrates including *C. tentans*, and for conducting static sediment toxicity tests with each species. These procedures were designed to enable direct comparison of test results for concurrent, single-species sediment assays for toxicity and bioaccumulation using *C. tentans* larvae, mayfly (*Hexagenia limbata*) nymphs, and fathead minnows.

**USEPA 1992** is a synthesis of information found in Hoke (1992) and Appendix A of USEPA (1994a), and represents the response of eight laboratories to a questionnaire sent by U.S. EPA's Office of Science and Technology, and Office of Research and Development, Washington, DC, regarding procedures used for conducting sediment toxicity tests for effects on survival and growth of *C. tentans* or *C. riparius*.

**RIZA 1993** is the draft procedure for culturing *C. riparius* and for conducting sediment-toxicity tests, developed by the Institute for Inland Water Management and Waste Water Treatment (RIZA; Lelystad, the Netherlands) for consideration by the Organization for Economic Cooperation and Development as an OECD guideline for testing of chemicals. See "RIZA 1993" in list of references.

**USEPA 1993** represents Call *et al.*, 1993. This unpublished report gives the draft method for culturing *C. tentans* and conducting sediment toxicity tests with this species, that was prepared for and by the USEPA.

**BBA/IVA 1994** is the procedure used for an international comparative assay with pesticides and *C. riparius*. See "BBA/IVA 1994" in list of references.

**Sheffield 1994** is the unpublished standard operating procedures used by C. Naylor and co-workers of the Dept. of Animal and Plant Sciences, University of Sheffield, Sheffield, U.K.

**USEPA 1994a** is the published methods for culturing *C. tentans* and other freshwater invertebrates, and for conducting sediment toxicity tests, developed by USEPA (principal authors, C.G. Ingersoll, G.T. Ankley, G.A. Burton, F.J. Dwyer, T.J. Norberg-King, and P.V. Winger). See "USEPA 1994a" in list of references.

## 1. Reference Toxicant(s), Test Type, Duration, and Frequency of Use

| Document <sup>a</sup> | Species                                   | Reference Toxicant(s)                                                  | Test Type                       | Test Duration   | Frequency of Use     |
|-----------------------|-------------------------------------------|------------------------------------------------------------------------|---------------------------------|-----------------|----------------------|
| USEPA 1984            | <i>C. tentans</i>                         | none                                                                   | none                            | --              | --                   |
| USFWS 1990            | <i>C. riparius</i>                        | none                                                                   | none                            | --              | --                   |
| ASTM 1991             | <i>C. tentans</i> ,<br><i>C. riparius</i> | none                                                                   | none                            | --              | --                   |
| NWRI 1992             | <i>C. riparius</i>                        | CuSO <sub>4</sub>                                                      | static, water only <sup>b</sup> | 48 h            | monthly <sup>c</sup> |
| OME 1992              | <i>C. tentans</i>                         | Cd, Cu <sup>d</sup>                                                    | water only <sup>d</sup>         | NI <sup>e</sup> | NI                   |
| USEPA 1992            | <i>C. tentans</i> ,<br><i>C. riparius</i> | NI                                                                     | NI                              | NI              | NI                   |
| RIZA 1993             | <i>C. riparius</i>                        | K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> ,<br>3,4-dichloroaniline | water only                      | 96 h            | ≥2×/year             |
| USEPA 1993            | <i>C. tentans</i>                         | CuSO <sub>4</sub> , KCl                                                | static, water only              | 96 h            | monthly              |
| BBA/TVA 1994          | <i>C. riparius</i>                        | none                                                                   | none                            | --              | --                   |
| Sheffield 1994        | <i>C. riparius</i>                        | none                                                                   | none                            | --              | --                   |
| USEPA 1994a           | <i>C. tentans</i>                         | NaCl, KCl,<br>Cd, Cu <sup>f</sup>                                      | static, water only              | 96 h            | monthly <sup>g</sup> |

<sup>a</sup> See preceding page for correct citation.

<sup>b</sup> Midge larvae are exposed to a range of concentrations of the reference toxicant dissolved in fresh water; no sediment is present in the test.

<sup>c</sup> Chronic reference toxicity tests with sediment are performed biannually, using CuSO<sub>4</sub> and CdCl<sub>2</sub>.

<sup>d</sup> The use of a positive control test, conducted as a water only assay using cadmium and/or copper, is recommended. No procedural specifics for performing reference toxicity tests using *C. tentans* are given.

<sup>e</sup> NI = Not indicated.

<sup>f</sup> Sodium chloride, potassium chloride, cadmium chloride, and copper sulphate are suitable for use. It might be unrealistic to test more than one or two reference toxicants routinely (i.e., monthly).

<sup>g</sup> Ideally, tests with reference toxicants should be conducted in conjunction with sediment toxicity tests.

## 2. Number of Organisms per Vessel, Age and Instar at Start of Test, Vessel Type and Size, Volume of Test Solution, and Number of Replicates per Treatment

| Document    | Species            | No. per Vessel    | Age at Start | Instar at Start | Test Vessel             | Volume of Solution | No. of Replic. |
|-------------|--------------------|-------------------|--------------|-----------------|-------------------------|--------------------|----------------|
| NWRI 1992   | <i>C. riparius</i> | 15                | 6-d old      | second          | 250-mL bkr <sup>a</sup> | 200 mL             | 3 to 5         |
| RIZA 1993   | <i>C. riparius</i> | NI <sup>b</sup>   | NI           | NI              | NI                      | NI                 | NI             |
| USEPA 1993  | <i>C. tentans</i>  | NI                | NI           | second          | 250-mL bkr              | 150 mL             | NI             |
| USEPA 1994a | <i>C. tentans</i>  | ≥ 10 <sup>c</sup> | NI           | third           | 250-mL bkr <sup>d</sup> | ≥ 100 mL           | ≥ 3            |
|             |                    | 1 <sup>c</sup>    | NI           | third           | 30-mL cup <sup>e</sup>  | 20 mL              | ≥ 10           |

<sup>a</sup> Glass beaker.

<sup>b</sup> NI = Not indicated.

<sup>c</sup> Reference toxicity tests can be conducted using one organism/vessel, or ≥ 10 organisms/vessel. Some laboratories have observed low control survival when more than one midge/vessel is used in "water only" tests.

<sup>d</sup> Glass beaker, covered with glass or plastic.

<sup>e</sup> Plastic cup, covered with glass or plastic.

## 3. Source and Hardness of Water Used in Test, and Variables Monitored

| Document    | Species            | No. per Vessel             | Water Source                                         | Variables Monitored <sup>a</sup> | Monitoring Frequency   |
|-------------|--------------------|----------------------------|------------------------------------------------------|----------------------------------|------------------------|
| NWRI 1992   | <i>C. riparius</i> | 15                         | dechl. tap <sup>b</sup>                              | DO pH cond                       | start and end          |
| USEPA 1993  | <i>C. tentans</i>  | NI <sup>c</sup>            | NI                                                   | NI                               | NI                     |
| RIZA 1994   | <i>C. riparius</i> | NI                         | NI                                                   | NI                               | NI                     |
| USEPA 1994a | <i>C. tentans</i>  | ≥ 10,<br>or 1 <sup>d</sup> | culture, well, site,<br>surface, recon. <sup>e</sup> | pH alk hard cond<br>temp DO      | start and end<br>daily |

<sup>a</sup> DO = dissolved oxygen; pH = hydrogen ion concentration; cond = specific conductivity; alk = total alkalinity; hard = total hardness; temp = temperature.

<sup>b</sup> Dechlorinated municipal tap water, same source and hardness as used for culturing midges.

<sup>c</sup> NI = Not indicated.

<sup>d</sup> Reference toxicity tests can be conducted using one organism/vessel, or ≥ 10 organisms/vessel.

<sup>e</sup> Reconstituted water. A recipe is provided for preparing suitable water with hardness 90 to 100 mg CaCO<sub>3</sub>/L.



#### 4. Temperature, Aeration, and Lighting During Test with Reference Toxicant

| Document    | Species            | No. per Vessel            | Water Temp. (°C) | Aeration Conditions | Lighting                                            |
|-------------|--------------------|---------------------------|------------------|---------------------|-----------------------------------------------------|
| NWRI 1992   | <i>C. riparius</i> | 15                        | NI               | NI                  | NI                                                  |
| RIZA 1993   | <i>C. riparius</i> | NI <sup>a</sup>           | NI               | NI                  | NI                                                  |
| USEPA 1993  | <i>C. tentans</i>  | NI                        | ambient          | NI                  | ambient                                             |
| USEPA 1994a | <i>C. tentans</i>  | ≥ 10 <sub>b</sub><br>or 1 | 23               | none                | 16L:8D, ~500 to 1000 lux, wide-spectrum fluorescent |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> Reference toxicity tests can be conducted using one organism/vessel, or ≥ 10 organisms/vessel.

#### 5. Substrate Used in Test with Reference Toxicant

| Document    | Species            | No. per Vessel            | Description of Substrate | Size/Quantity of Substrate |
|-------------|--------------------|---------------------------|--------------------------|----------------------------|
| NWRI 1992   | <i>C. riparius</i> | 15                        | NI <sup>a</sup>          | NI                         |
| RIZA 1993   | <i>C. riparius</i> | NI                        | NI                       | NI                         |
| USEPA 1993  | <i>C. tentans</i>  | NI                        | no. 30 grade silica sand | light layer                |
| USEPA 1994a | <i>C. tentans</i>  | ≥ 10 <sub>b</sub><br>or 1 | sand                     | monolayer                  |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> Reference toxicity tests can be conducted using one organism/vessel, or ≥ 10 organisms/vessel.

## 6. Feeding During Test with Reference Toxicant

| Document    | Species            | No. per Vessel | Description of Food Used   | Quantity per Vessel                    | Feeding Frequency            |
|-------------|--------------------|----------------|----------------------------|----------------------------------------|------------------------------|
| NWRI 1992   | <i>C. riparius</i> | 15             | NI <sup>a</sup>            | NI                                     | NI                           |
| RIZA 1993   | <i>C. riparius</i> | NI             | NI                         | NI                                     | NI                           |
| USEPA 1993  | <i>C. tentans</i>  | NI             | Tetrafin™<br>goldfish food | 4 mg <sup>b</sup>                      | Day 0 only                   |
| USEPA 1994a | <i>C. tentans</i>  | ≥ 10<br>1      | Tetrafin™<br>Tetrafin™     | 5 mg <sup>c</sup><br>1 mg <sup>d</sup> | Days 0 and 2<br>Days 0 and 2 |

<sup>a</sup> NI = Not indicated.

<sup>b</sup> Organisms in each test vessel are fed 1.0 mL of a 4 mg/mL suspension of Tetrafin™.

<sup>c</sup> Organisms in each test vessel are fed 1.25 mL of a 4 mg/mL suspension of Tetrafin™.

<sup>d</sup> The single midge larva in each cup is fed 0.25 mL of a 4 mg/mL suspension of Tetrafin™.

## 7. Endpoints and Requirement for Valid Test Using Reference Toxicant

| Document    | Species            | No. per Vessel  | Biological Endpoint      | Statistical Endpoint   | Requirement for Valid Test                                           |
|-------------|--------------------|-----------------|--------------------------|------------------------|----------------------------------------------------------------------|
| NWRI 1992   | <i>C. riparius</i> | 15              | % survival               | 48-h LC50 <sup>a</sup> | mean survival ≥ 90% <sup>b</sup>                                     |
| RIZA 1993   | <i>C. riparius</i> | NI <sup>c</sup> | % survival               | 96-h LC50              | specified 95% CLs <sup>d</sup>                                       |
| USEPA 1993  | <i>C. tentans</i>  | NI              | % survival               | 96-h LC50              | NI                                                                   |
| USEPA 1994a | <i>C. tentans</i>  | ≥ 10<br>1       | % survival<br>% survival | 96-h LC50<br>96-h LC50 | mean survival ≥ 80% <sup>b</sup><br>mean survival ≥ 80% <sup>b</sup> |

<sup>a</sup> Median lethal concentration.

<sup>b</sup> For controls used in test with reference toxicant.

<sup>c</sup> NI = Not indicated.

<sup>d</sup> 95% confidence limits >20 mg/L, <75 mg/L for K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>; and >4 mg/L, <12.5 mg/L for 3,4-dichloroaniline.

## Appendix F

General Systematics of *Chironomus tentans* (Fabricius, 1805)<sup>53</sup>**Taxonomy and Phyletic Relationships**

*Chironomus tentans* is a large nonbiting midge (Order Diptera or true flies) with two aquatic immature stages and a short terrestrial adult stage. The species belongs to the Family Chironomidae (=Tendipedidae), Subfamily Chironominae, Tribe Chironomini, and Subgenus *Camptochironomus*. For the nonspecialist, chironomid species and especially their larvae are difficult to identify because of a lack of descriptive taxonomic material; in addition, most species are described only for the adult or imago stage (Pinder, 1983).

The general morphology of larvae, pupae and adults representing *Chironomus* spp. is illustrated in Figures F.1 and F.2. Figure F.3 shows the distinguishing features of larval and adult *C. tentans*.

**Distribution**

*C. tentans* is a holarctic insect. The species is widespread in Europe, from Austria in the South, to England in the West, Scandinavia in the north, and Russia in the east (Acton and Scudder, 1971). In North America, it has been recorded in Canada within British Columbia, Alberta, Saskatchewan, Manitoba, Ontario and Quebec; and in the United States within Massachusetts, New York, Michigan, Illinois, Wisconsin, Minnesota, Iowa, Idaho, North Dakota, South Dakota, Colorado, Montana, and Utah (Townes, 1945).

**Ecology**

The family Chironomidae is the most ubiquitous and usually most abundant insect group in all types of freshwater, often reaching densities of many thousand per square metre (Pinder, 1983). In North America and Europe, larvae of *C. tentans* are found in shallow, slow-flowing or stagnant water that is often semipermanent, with emergent vegetation and abundant decaying organic matter such as dead leaves and dead algae (Acton and Scudder, 1971; Curry, 1962; Topping, 1971; Townes, 1945). This species has also been recorded from shallow brackish water in northern Europe (Acton and Scudder, 1971), and is thought to be often indicative of moderate organic pollution (Townes, 1945). In British Columbia, *C. tentans* larvae have been found in lakes at depths of 0 to 2 metres (Topping, 1971). They occurred most frequently in soft ooze (particle size < 0.15 mm and organic carbon 1.9 to 5.4%) and detritus. Larvae have been found at temperatures ranging 0 to 23°C, dissolved oxygen 0.2 to 8.2 mg/L, pH 8.0 to 9.2, and conductivity 481 to 4136 µmho/cm. Hydrogen sulphide concentrations in water appeared to limit the occurrence of the species, as larvae were absent where concentrations exceeded 0.3 mg/L (Topping, 1971).

<sup>53</sup> Prepared by P. Hamr, NWRI Rivers Research Branch, Environment Canada, Canada Centre for Inland Waters, Burlington, ON.

Larval stages live in the top layer of sediment (upper 10 cm), and build tubes or cases into which they are anchored by their caudal extremities. They draw food particles into the tubes, but also feed in the immediate vicinity of the tubes (ASTM, 1991a; 1995a). *C. tentans* larvae consume a great variety of food particles, including *Zygema*, *Cladophora*, *Spirogyra*, diatoms, desmids, and crushed *Lemna* (Sadler, 1935). Silt might also be ingested along with food particles (Sadler, 1935). Chironomid larvae are important components in the diets of macroinvertebrates, fish, and waterfowl (Townsend *et al.*, 1981).

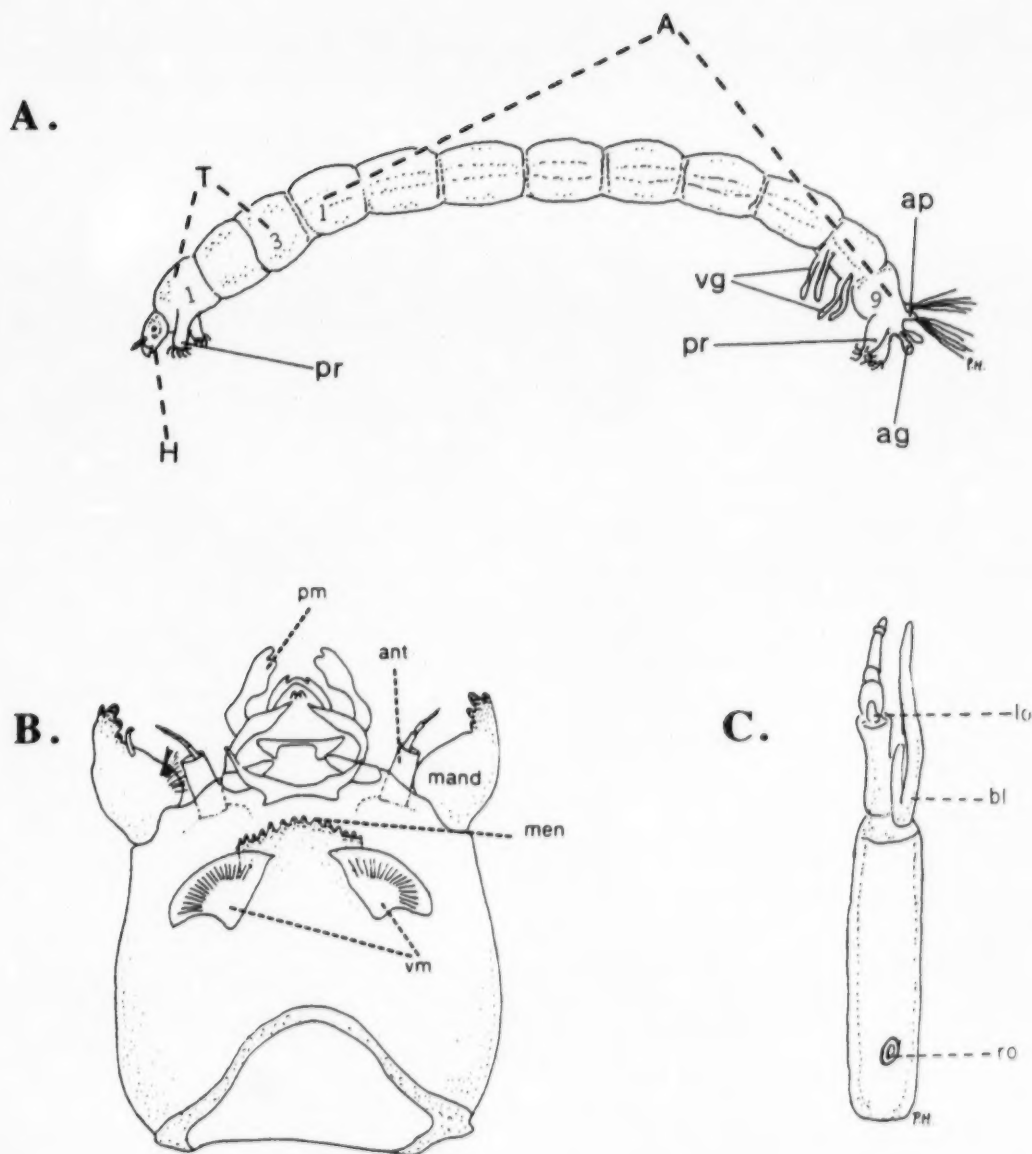
*C. tentans* co-occurs with other species of chironomids including *C. riparius* as well as *Glyptotendipes* spp., *Anatopynia* spp., and *Limnochironomus* spp. The absence of *C. tentans* larvae in British Columbia lakes at depths greater than 2 m is thought to be indicative of interspecific competition (Topping, 1971).

### ***Life Cycle and Reproductive Biology***

The life cycle of *C. tentans* is composed of the following four distinct stages: egg, larva, pupa, and adult (imago). The larval and pupal stages are aquatic. Adults can be collected from spring to autumn (April-September) (Townes, 1945). Adult males are distinguished from females by large plumose antennae and much thinner abdomens, with visible genitalia consisting of paired claspers on the posterior tip of the abdomen (Figure F.3f). Adult females weigh about twice as much as males and approximately 30% of their weight is due to eggs (ASTM 1991a; 1995a). Mating, which is initiated by the male, occurs in flight and takes approximately 20 seconds (Townsend *et al.*, 1981). Males are capable of copulating with more than one female. Spermatozoa are stored in the female's paired spermatheca until oviposition. At 24 to 48 h after mating, females deposit a single clear, gelatinous egg mass directly into the water. The gelatin is hygroscopic and the egg mass rapidly expands into a characteristic "C" shape. An egg mass contains approximately 2300 eggs and will hatch in 2 to 4 days at 23°C (ASTM, 1995a).

The larval stage consists of four instars. The first instar, which is 0.6 to 1.2 mm long and creamy white in colour, remains in/on the gelatinous mass initially. The head capsule width of first instars is normally within the range 0.09 to 0.13 mm. Tube construction within surficial sediment is initiated by the first instars at about 2 to 3 days after hatching. The second instar is pink due to hemoglobin production, measures 2.0 to 4.5 mm in length, and has a head capsule width of 0.18 to 0.23 mm. The third and fourth instars are both bright red. The third instar measures 6 to 12 mm and its head capsule is 0.33 to 0.45 mm wide. The fourth instar measures 12 to 24 mm, has a head-capsule width of 0.63 to 0.71 mm, and can be distinguished by a black longitudinal strip extending from the frontal to the clypeus on the head capsule (Townsend *et al.*, 1981; Norberg-King, 1994).

Metamorphosis occurs within the larval tube. The pupa leaves the tube after the larval-pupal molt, and swims on the sediment surface. The pupa is black and the most prominent changes in morphology are the development of respiratory and locomotory organs and appendages (Townsend *et al.*, 1981). The pupal stage lasts about 3 days, after which the pupa rises to the surface of the water column and emergence occurs. The emergence process takes about 15 seconds. Overall, larvae will pupate and emerge as adults after about 21 days at 23°C. The adult stage lasts 3 to 5 days, during which the adults reproduce.

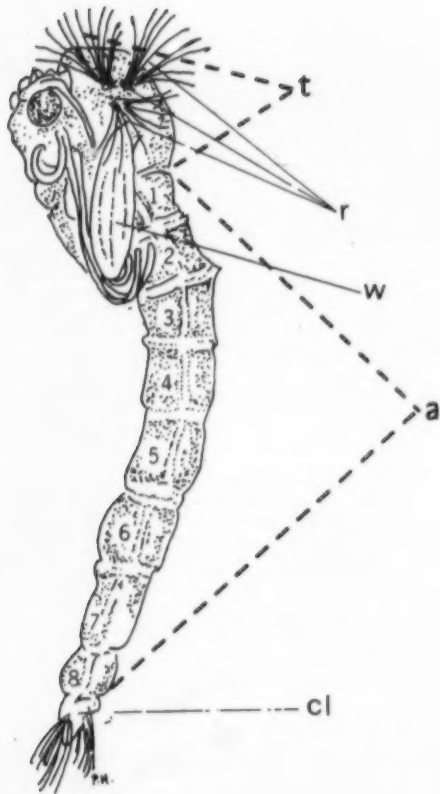


- A. Lateral view of a chironomid larva.  
 H, head capsule; T1-3, thoracic segments; A1-9, abdominal segments; ap, anal papillae (procerci); pr, prolegs (parapods); vg, ventral gills (tubules); ag, anal gills (tubules).  
 B. Ventral view of a Chironomini head capsule.  
 ant, antenna; mand, mandible; men, mentum (labial plate); vm, ventromental (paralabial) plate; pm, premandible.  
 C. Antenna. ro, ring organ; bl, blade; lo, Lauterborn organ.

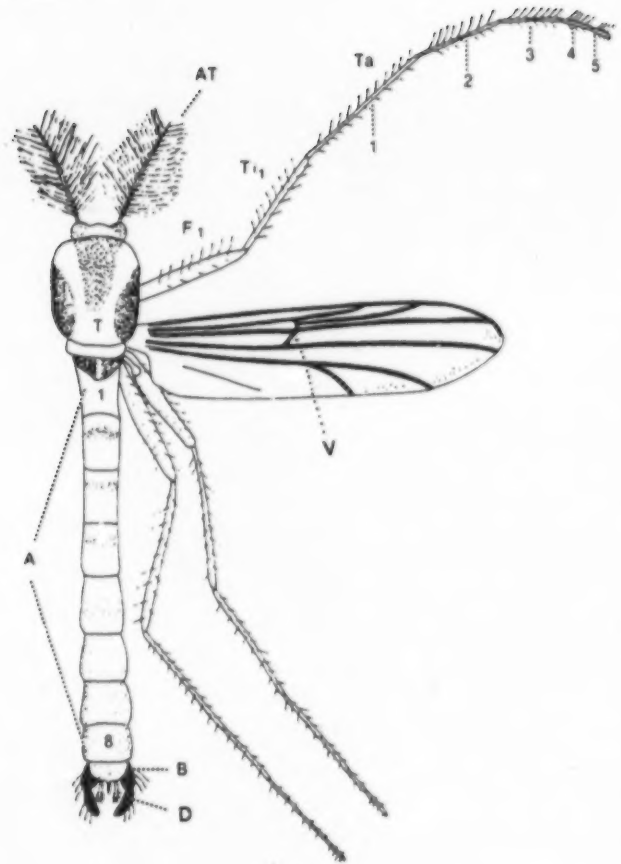
**Figure F.1** General Morphology of *Chironomus* spp. Larvae (from Coffman, 1984 and Cranston and Reiss, 1983)



A.



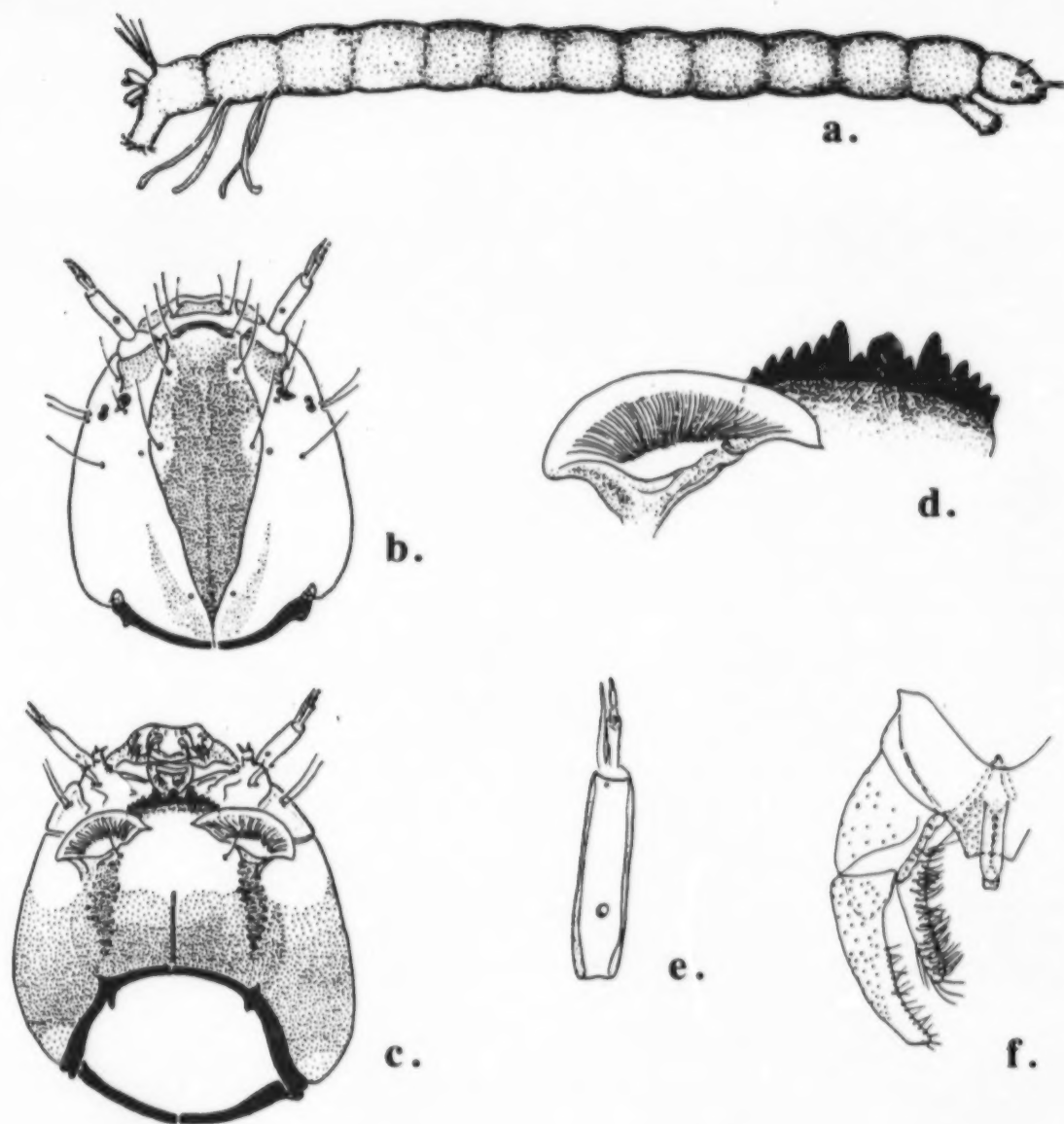
B.



A. Lateral view of a *Chironomus* sp. pupa.  
t, thorax; a, abdomen; r, respiratory organs; w, wing sheat; cl, caudal lobe.

B. Dorsal view of adult male chironomid (Chironomini).  
AT, antenna; T, thorax; A, abdominal tergites 1-8; B, basistyle of genitalia; D, dististyle of genitalia; F<sub>1</sub>, femur of foreleg; Ti<sub>1</sub>, tibia of foreleg; Ta 1-5, tarsal segments of foreleg; V, radial-medial cross-vein.

**Figure F.2** General Morphology of *Chironomus* spp. Pupae and Imagos (from Coffman, 1984)



LARVA: a. larva, lateral view; b. head capsule, dorsal view; c. head capsule, ventral view; d. mentum and ventromental plate; e. antenna.

ADULT: f. male genitalia.

**Figure F.3** *Chironomus tentans* (Fabricius, 1805) (from Malloch, 1917; Townes, 1945; and Kiknadze *et al.*, 1991)

***Chironomus tentans* (Fabricius, 1805)**

**Figure F.1**      **Diagnosis** (after Johannsen, 1937a; b; Townes, 1945; Curry, 1962; and Coffman, 1984)

**Larva:** 18-27 mm long with three main divisions: sclerotized head, thorax (3 segments) and abdomen (9 segments). Abdomen with 4 long ventral gills and 2 pairs of anal gills (Figure F.2a). Anal papillae (procerci) with pencil of 6 hairs. Labrum with bristles, spines, and pectinate scales. Epipharyngeal plate with 13 - 15 teeth. Antenna is approximately 0.25 mm long with ring organ on basal third of first segment. Antennal blade extends from base of second segment to base of fifth segment (Figure F.2e), and Lauterborn organs are located at the base of the third segment. Mandibles with preapical comb composed of approximately 10 bristles, four black teeth, short stout pale accessory tooth, and 2-3 serrations on the inner pale basal margin. Maxillae twice as wide as long and palpus twice as long as wide with usual sense papillae. Labial plate has 13 black teeth with second lateral teeth taller than the trilobed middle tooth (Figure F.2d). Epipharyngeal plate has 13 lobed teeth varying from large to small. A short gill-like process is present on the latero-posterior margin of the tenth segment.

**Pupa:** 10-12 mm long, red in colour just after pupation but becoming darker later. Sternites 2-4 with minute spinules arranged in rows in groups of 2-5 or more on latero-anterior portion. Segments 7 and 8 with 3 and 4 lateral filaments, respectively. Spur on segment 8 composed of 5-9 fused spines and fringe of caudal lobe with about 125 filaments. Tubular gill-like evagination on each side of the conjunctiva. Cephalic tubercles are cone-shaped, and pupal spur is composed of 6-8 fused spines.

**Adult:** Ground colour is pale greenish to light brown. Wing 5.8 mm long with long brown anterior veins, darker radial-medial cross-vein, and pale posterior veins. Antennal ratio 3.8. Leg ratio 1.2, legs are pale brown at bases with brown apices. Body is very stout conspicuous mesoscutal stripe and dark abdomen. Frontal tubercles conical and of moderate size; clypeus rather small; middle portion of pronotum rather strongly widened and produced. Fore tarsus without a beard with exceptionally short clothing hairs. Male genitalia (hypopygium) distinctive (Figure F.2f), very large and heavy. Female is identified by colouration as well as strongly-produced middle portion of the pronotum.

## Appendix G

# General Systematics of *Chironomus riparius* (Meigen, 1804)<sup>54</sup>

## Taxonomy and Phyletic Relationships

*Chironomus riparius* is a nonbiting midge (Order Diptera or True flies ) with two aquatic immature stages and a short terrestrial adult stage. The species belongs to the Family Chironomidae (=Tendipedidae), Subfamily Chironominae, Tribe Chironomini, and subgenus *Chironomus*. *Chironomus riparius* has been found to be synonymous with *Chironomus thummi* Kieffer (Credland, 1973).

Figures F.1 and F.2 in Appendix F illustrate the general morphology of larvae and adults representing *Chironomus* spp., including *C. riparius*. Figure G.1 shows the distinguishing taxonomic features of larval and adult *C. riparius*.

## Distribution

*C. riparius* is a holarctic species. In North America, it has been recorded in Canada within the Provinces of Alberta, Saskatchewan, Manitoba, Ontario, Quebec, Newfoundland, and the Northwest Territories. In the United States, the species has been recorded in Massachusetts, New Hampshire, Rhode Island, New Jersey, New York, Illinois, Ohio, Pennsylvania, Michigan, Minnesota, District of Columbia, Maryland, North Carolina, South Carolina, Indiana, Iowa, Kansas, Virginia, Missouri, Nevada, Colorado, and Alaska (Townes, 1945; Curry, 1962; Rasmussen, 1984). This species is also widespread in Europe, and is common in Austria, Belgium, France, Britain, Germany, Italy, and Scandinavia (Townes, 1945; Curry, 1962; Credland, 1973). Townes (1945) also reported the distribution of this species in Argentina.

## Ecology

The tubiculous larvae of *C. riparius* frequently inhabit eutrophic lakes, ponds, and streams; and reportedly live in mudbottom littoral habitats to depths up to 1 m (Rasmussen, 1984; ASTM, 1995a). According to Curry (1962), the larvae construct long burrows or tubes in soft mud and have been collected from particulate marl, fibrous peat, plant remains, and muck, at depths of up to 3 to 6 metres. They are mainly deposit feeders, frequently extending their anterior ends outside tubes to feed on the surficial layer of sediment at the sediment/water interface (Rasmussen, 1984; ASTM, 1995a). As with *C. tentans*, this species has been found to be tolerant of organic enrichment and is therefore considered to be a ubiquitous indicator of organic pollution (Townes, 1945; Gower and Buckland, 1978). *C. riparius* appears to have a wide tolerance for sediment grain size, and has been found in the field at temperatures from 0 to 33°C, pH ranging from 5 to 9, and dissolved oxygen concentrations as low as 1 mg/L (ASTM, 1991a; 1995a).

<sup>54</sup> Prepared by P. Hamr, NWRI Rivers Research Branch, Environment Canada, Canada Centre for Inland Waters, Burlington, ON.

### ***Life Cycle and Reproductive Biology***

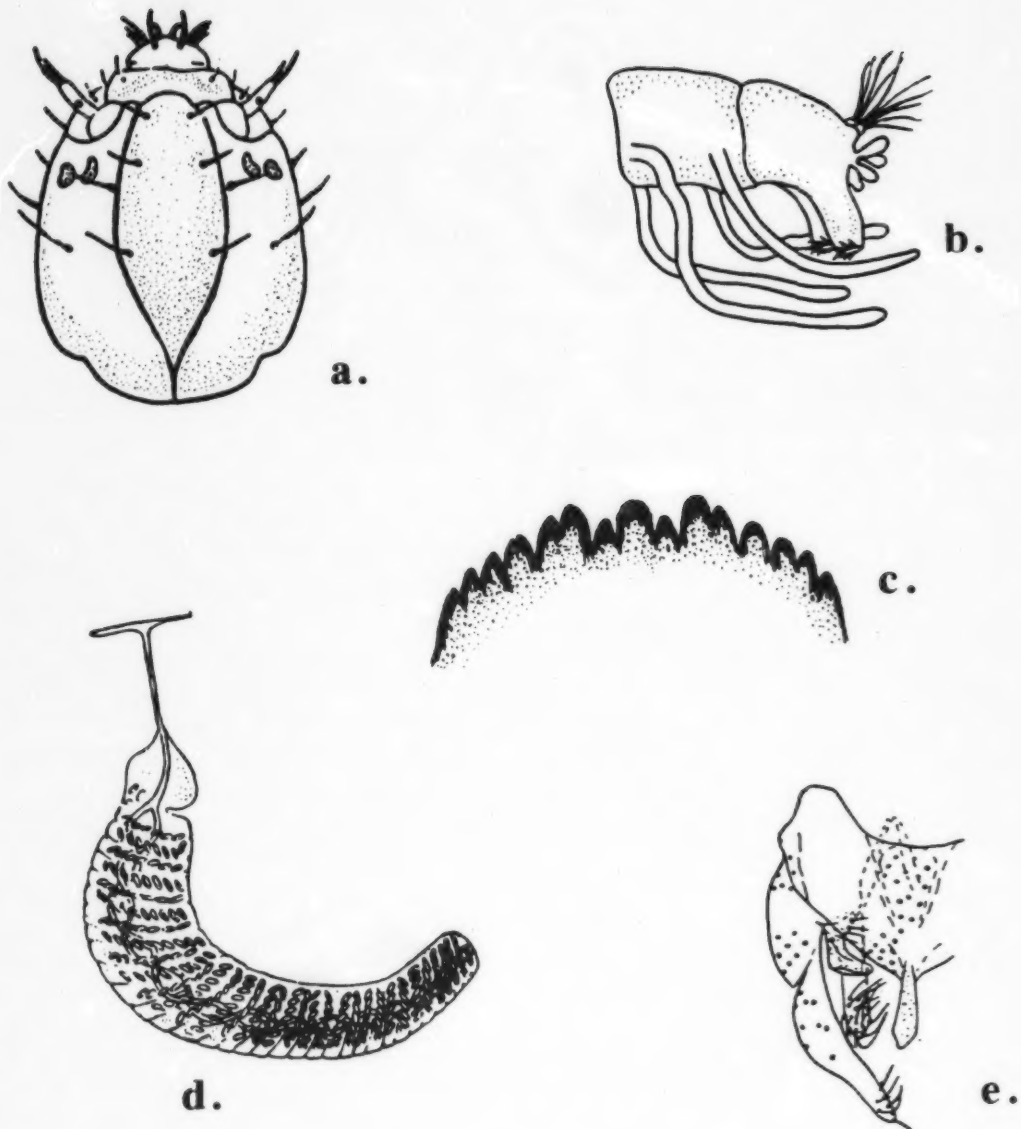
The life cycle of *C. riparius* is composed of four distinct stages: the egg stage, the larval stage, the pupal stage, and the adult (imago) stage. The larval and pupal stages are aquatic. Adults are found throughout the summer, but are most abundant during the early spring (Townes, 1945; Curry, 1962). Males can be distinguished from females by large plumose antennae and thin abdomens, with visible genitalia on terminal segments of the abdomen. The reproductive behaviour of *C. riparius* is similar to that of *C. tentans*. Mating occurs during swarming, and after 1 to 2 days the female lays a "C" shaped gelatinous egg mass (RIZA, 1993) (Figure G.1d). The egg mass contains approximately 150 to 600 eggs, and will hatch in about 2 to 6 days depending on water temperature (Curry, 1962; ASTM, 1991a, 1995a; RIZA, 1993).

Each instar stage lasts about 4 to 7 days. The head capsule width of the various instars are as follows: first instar, 0.07 to 0.12 mm; second instar, 0.13 to 0.24 mm; third instar, 0.26 to 0.40 mm; and fourth instar, 0.43 to 0.60 mm (RIZA, 1993; Day *et al.*, 1994). Most growth appears to occur in the fourth instar (Rasmussen, 1984), which is blood red with a yellowish brown head and black eye spots (Curry, 1962). Sexual dimorphism occurs in the fourth instar, with larval male chironomids weighing, on the average, 29.4 % less than females (Day *et al.*, 1994).

After the fourth instar, the larvae pupate and emergence occurs immediately after pupae swim to the water surface, with male chironomids emerging earlier (by ~2 days) than females (RIZA, 1993). The adult females are larger and darker than the males.

The total life cycle of *C. riparius* is 15 to 30 days on average, and adults of both sexes are estimated to live 4 to 11 days (Curry, 1962; RIZA, 1993; ASTM, 1995a). In prairie ponds of Alberta, the species exhibits a univoltine life cycle (Rasmussen, 1984). Overwintering occurs in the fourth instar stage; and pupation, emergence, and oviposition occur mainly in May. In England, the species is multivoltine, with at least five generations in a year (Gower and Buckland, 1978). Development is dependent on temperature (28 d at 18°C and 49 d at 12°C), and all stages of the life cycle can be present throughout summer and autumn.





LARVA: a. head capsule, dorsal view; b. caudal segments; c. mentum; d. egg mass.  
 ADULT: e. male genitalia.

**Figure G.1** *Chironomus riparius* (Meigen, 1804) (from Townes, 1945 and Usinger, 1963)

*Chironomus riparius* (Meigen, 1804)

**Figure G.1**      **Diagnosis** (after Johannsen, 1937b; Townes, 1945; Curry, 1962; and Coffman, 1984)

**Larva:** 16 mm long with three main divisions: sclerotized head, thorax (3 segments), and abdomen (9 segments). Two pairs of ventral gills on eleventh segment, but no lateral gill-like processes on tenth segment (Figure G.1b). Anal papillae (procerci) with pencil of 6 hairs. Antenna 0.18 mm long, with blade extending from base of second to middle of fifth segment. Ring organ in basal fourth of first segment. Mandible with preapical comb, compound brush with 3 main trunks, and 2 setae near convex margin. Three mandibular lateral teeth with black apical tooth and innermost lateral tooth not as dark as other two teeth. Labial plate with 13 dark, lobated teeth; median tooth trilobed, often appearing to be three separate teeth, and broader than high. First laterals taller than median tooth and closely adjacent to second laterals. Paralabial plates are nearly three times as wide as long. Epipharyngeal plate has 14 long, sharply pointed uniform teeth.

**Pupa:** 10 mm long, brownish-yellow with margins of wing pads, thoracic appendages, lateral margins of abdominal segments, and apical appendages blackened. Cephalic tubercles acute with preapical bristle. Notum of thorax nodulose, abdominal tergites with usual spines and spinules. Thoracic respiratory organ with numerous white filaments. Spur of segment 8 consists of 3-4 fused spines. Segments 5-7 with 4 flat filaments each, segment 8 with 5 filaments. Caudal lobe dark brown with fringe of more than 100 filaments.

**Adult:** Ground colour of pale to dark brown with red-brown to blackish-brown thoracic markings. Wing 3.7 mm long, anterior wing vein brown, radial-medial cross-vein somewhat darker, posterior wing veins unpigmented. Antennal ratio 3.3. Leg ratio 1.6, legs pale green to light brown toward bases and apices. Fore tarsus without beard. Abdomen brown to blackish, apical portion of tergites pruinose and pale. Female similar in colour to male. Male genitalia (hypopygium) are distinctive, with broad short superior appendage and narrow anal point (Figure G.1e).

*C. riparius* co-occurs with other species of chironomids including *C. decorus*, *C. plumosus*, *C. tentans*, *C. staegeri*, and *Glyptotendipes paripes* (Curry, 1962; Rasmussen, 1984).

## Appendix H

# Logarithmic Series of Concentrations Suitable for Toxicity Tests<sup>55</sup>

Column (Number of concentrations between 10.0 and 1.00, or between 1.00 and 0.10)<sup>56</sup>

| 1    | 2    | 3    | 4    | 5    | 6    | 7    |
|------|------|------|------|------|------|------|
| 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| 3.2  | 4.6  | 5.6  | 6.3  | 6.8  | 7.2  | 7.5  |
| 1.00 | 2.2  | 3.2  | 4.0  | 4.6  | 5.2  | 5.6  |
| 0.32 | 1.00 | 1.8  | 2.5  | 3.2  | 3.7  | 4.2  |
| 0.10 | 0.46 | 1.00 | 1.6  | 2.2  | 2.7  | 3.2  |
|      | 0.22 | 0.56 | 1.00 | 1.5  | 1.9  | 2.4  |
|      | 0.10 | 0.32 | 0.63 | 1.00 | 1.4  | 1.8  |
|      |      | 0.18 | 0.40 | 0.68 | 1.00 | 1.3  |
|      |      | 0.10 | 0.25 | 0.46 | 0.72 | 1.00 |
|      |      |      | 0.16 | 0.32 | 0.52 | 0.75 |
|      |      |      | 0.10 | 0.22 | 0.37 | 0.56 |
|      |      |      |      | 0.15 | 0.27 | 0.42 |
|      |      |      |      | 0.10 | 0.19 | 0.32 |
|      |      |      |      |      | 0.14 | 0.24 |
|      |      |      |      |      | 0.10 | 0.18 |
|      |      |      |      |      |      | 0.13 |
|      |      |      |      |      |      | 0.10 |

<sup>55</sup> Modified from Rochinni *et al.* (1982).

<sup>56</sup> A series of five (or more) successive concentrations should be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by weight (e.g., mg/kg) or weight-to-volume (e.g., mg/L). As necessary, values can be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations should not be used, since such will provide poor resolution regarding the confidence limits surrounding any threshold-effect value calculated.